

Hydrogel Encapsulation for the Culture of Bone Marrow Stromal Cells

By

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ABSTRACT

In tissues, cells are encapsulated within extracellular matrix (ECM) and biopolymer hydrogels serve to mimic ECM *in vitro* by providing a hydrated three-dimensional environment widely investigated as scaffold materials for tissue engineering applications. Such hydrogels can be used to deliver cells to a specific site *in vivo* before being released by a process of degradation. In order to produce hydrogel cell culture substrates that are fit for purpose, it is important that the mechanical properties are well understood, not only at the point of cell seeding but also throughout the culture period. In this thesis, the change in mechanical properties of three biopolymer hydrogels 0.5%, 1% and 5%w/v alginate, 0.5%, 1% and 5% w/v low methoxyl pectin and 0.5%, 1% and 2% w/v gellan gum have been assessed in cell culture conditions. The samples were seeded with rat bone marrow stromal cells and were cultured in osteogenic media. Acellular samples were incubated in standard cell culture media. The rheological properties of the gels were measured over a culture period of 28 days and it was found that the gels degraded at very different rates. The degradation occurred most rapidly in the order alginate > low methoxyl pectin > gellan gum hydrogels. The ability of each hydrogel to support differentiation of bone marrow stromal cells to osteoblasts was also verified by evidence of mineral deposits in all three of the materials. Further to this study, the effect of nanocrystalline hydroxyapatite on the mechanical properties of 0.5%, 1% and 1.5% w/v gellan gel has been studied through rheological and compression testing. The results reported a decrease in the modulus of 0.5% gellan caused by agglomeration of HA particles and a relatively higher modulus in higher concentration of gellan gel. The surface morphology of the composite and the homogeneous dispersion of nanoparticles in gellan matrix have been investigated through scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Calcium release study further suggested the use of hydroxyapatite as a crosslinker for the hydrogels.

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LIST OF ABBREVIATIONS

ALP: Alkaline Phosphatase

BM: Bone marrow

CD: Cyclodextrin

DE: Degree of Esterification

dH₂O: Deionized Water

DMEM: Dulbecco's modified Eagle's Medium

ECM: Extracellular Matrix

EG: Ethylene glycol

EGDMA: Ethylene glycol dimethacrylate

ESC: Embryonic Stem Cell

FBS: Foetal Bovine Serum

HA: Hydroxyapatite

HEMA: Hydroxyethyl methacrylate

HM: High-Methoxyl Pectin

LM: Low-Methoxyl Pectin

MBAAm: Methylene-bis-acrylamide

MSC: Mesenchymal Stem Cells

MW: Molecular Weight

P(...): Poly(....)

PAAc: Poly(acrylic acid)

PAAm: Poly acrylamide

PAGE: Polyacrylamide gel electrophoresis

PBO: Poly(butylene oxide)

PBS: Phosphate Buffered Saline

PCL: Polycaprolactone

PEG: Poly(ethylene glycol)

PF: Propylene fumarate

PGEMA: Poly(glucosylethyl methacrylate)

PHB: Poly(hydroxyl butyrate)

PHPMA: Poly(hydroxypropyl methacrylamide)

PLA: Poly(lactic acid)

PLGA: Poly(lactic-co-glycolic acid)

PPO: Poly(propylene oxide)

rBMSC: Rat Bone Marrow Stromal Cells

SC: Stem Cell

sSC: Somatic Stem Cell

Tri-calcium phosphate: TCP

α -MEM: Alpha Minimum Essential Medium Eagle

1.INTRODUCTION

Bone defects resulting from tumours, diseases, infections, trauma and skeletal disorders cause significant discomfort and disability to patients. Traditional methods of grafting as in autografts, allografts and other therapies not only have not been able to prevail over the high demands for bone repair but also have proven to carry significant side-effects (Betz 2002). Tissue engineering has offered a new approach for bone repair with the development of suitable scaffolds for three-dimensional (3D) culture of osteogenic cells to succeed treatment of bone defects. A number of biomaterials have been used in tissue engineering (He & Liu 2010) of bone (Langer & Vacanti 1993;Salgado et al. 2004) ranging from synthetically derived materials to naturally occurring biopolymers (Hubbell 1995;Langer & Tirrell 2004). One limiting factor of traditional high-density 3D constructs is that it is hard to maintain the viability of the cells located in the centre of the scaffold and this is due to the poor exchange of nutrients and dissolved gases (Cartmell et al. 2003;Glowacki & Mizuno 2008). Biopolymer hydrogels in particular, have received much attention as potential tissue engineering scaffolds (Hunt & Grover 2010). They have been combined with solid scaffolds (Gkioni et al. 2010), injected into the site (Jeong & Gutowska 2001), prepared as a cell sheet (Kikuchi & Okano 2005) and also used in organ printing (Boland et al. 2003;Fedorovich et al. 2007). These biopolymer hydrogels offer several advantages over synthetic polymers and inorganic scaffolds. Biopolymer hydrogels provide a 3D environment that is morphologically similar to the extracellular matrix (ECM) of native tissue. The mild sol-gel transitions of these gels facilitate 3D encapsulation of cells (Mao et al. 2001). Cells, growth factors and other bioactive

compounds can be homogeneously suspended within the hydrogel matrix. The high water content of these biopolymers allows rapid diffusion of hydrophilic nutrients and metabolites whilst producing little to no cytotoxic by-products (Boland et al. 2003;Fedorovich et al. 2007). Biopolymer hydrogels can also be designed to exhibit similar mechanical properties as a variety of tissues and can gradually degrade within biological systems.

In an ideal situation, the rate of degradation of the scaffold should be equivalent to the rate of new tissue formation, so the mechanical integrity of the tissue is maintained during healing (Uebersax et al. 2006). Since the rate of tissue formation varies to a great extent, there is a need for different hydrogels with varying rates of degradation. The polysaccharide, alginate has been widely investigated as a promising 3D tissue culture substrate (Hunt et al. 2010;Paige et al. 1995); however other polysaccharides with similar hydrogel forming properties such as pectin and gellan gum have surprisingly received much less interest. Like alginate, both pectin and gellan gum undergo ionotropic gelation in the presence of divalent cations (calcium most commonly in cell encapsulation). However, gellan gum can also be crosslinked with monovalent cations. This further explains the reason that gellan gum can be crosslinked in the presence of culture media (contains monovalent salts) without the addition of further salts (Smith et al. 2007).

Alginate is comprised of β -D-mannuronate (M) and α -L-guluronate (G) organized into blocks of M and G residues as well as alternating sequences whereas pectins are complex polysaccharides. They consist of long sequences of 1,4-linked-D-galacturonate residues with small fractions of other sugars along the chains which include xylose,

glucose, rhamnose, arabinose, and galactose. Pectins also contain other non-sugar groups that can include acetic acid, phenolic acids and most notably methanol, esterified on the galacturonate residues. The extent of methylation is an important structural characteristic of pectins especially with regards to the gelation properties. The degree of esterification (DE) is defined as the percentage of galacturonate esterified with methanol. If more than 50% of the carboxyl groups on the galacturonate are methylated, the pectins are described as high-methoxyl (HM) pectins, and less than 50% methylation are called low-methoxyl (LM) pectins (Sungthongjeen et al. 2004).

Gelation of alginate and LM pectin depends on the interaction between divalent cations and blocks of guluronate and de-esterified galacturonate residues respectively (Braccini & Perez 2001). The gelation mechanism of both alginate and LM pectin is described through the 'egg-box' model (Grant et al. 1973). However, there are subtle differences between the two (Fang et al. 2008). Gellan gum is a microbial exopolysaccharide produced by the aerobic fermentation of *Sphingomonas elodea* (Dai et al. 2008; Doner 1997). Gellan gum consists of repeating tetrasaccharide units of glucose, glucuronic acid, glucose and rhamnose residues (Chandrasekaran et al. 1988). At temperatures above 85°C, gellan gum polymers appear as a disordered coil, which forms a helical structure upon cooling, forming a weak gel. The addition of monovalent or divalent cations to the gellan gum solution allows the formation of a self-supporting hydrogel (Matricardi et al. 2009) and their mechanical properties and gelation temperature can be controlled by manipulation of the salt concentration and species (Ogawa 1996).

Hydroxyapatite [HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] is a bioceramic with a compositional similarity to bone mineral. It has been used as a coating and synthetic bone substitute material (Rosen et al. 2002). One of the drawbacks of this ceramic is that in particulate form it exhibits no mechanical integrity, and sintering reduces bioactivity which limits its use in hard tissue implants. However, if mixed with hydrogels, particulate can be bound in place and the mechanical properties exhibited by the gel enhanced (Fedorovich et al. 2007).

In this study, the change in mechanical properties of three biopolymer hydrogels: alginate, LM pectin and gellan gum, have been assessed in cell culture conditions. The ability of each hydrogel to support differentiation of bone marrow stromal cells (BMSCs) to osteoblasts was also verified by evidence of mineral deposits in all three hydrogels. Also, we hypothesized that the incorporation of HA in gellan gum hydrogels could improve the gel modulus that allows for the modification of the degradation rate of the gels.

2. BONE AND TISSUE ENGINEERING

Bone is formed through a series of events starting with recruitment of osteoprogenitor cells from surrounding tissues and further proliferation, followed by osteoblastic differentiation, matrix formation, and mineralization (Shin et al. 2003).

An increase in the aged population has given rise to a higher rate of musculoskeletal impairment, osteoarthritis, back and spine impairment and osteoporosis-related fractures as well as non-age related problems in need of surgical procedures. The traditional method of bone grafting that uses autologous bone causes severe pain associated with harvesting. Banked allogeneic and xenogeneic bone grafts show a high risk of disease transmission. Also, prosthetic implants (e.g. metallic hip and knee) have been the choice of the surgeons in orthopaedics, dental defect and craniofacial surgeries until today. Recently, a large amount of research has been undertaken in the field of bone tissue regeneration. A variety of biomaterials: such as titanium, polyethylene, or silicone, and bioactive ceramics as in HA, tri-calcium phosphate (TCP) or a mixture of the two are widely used for implantation and reconstruction as they offer controllable physical properties with no risk of disease transmission from the implant. However as there is a need of re-implantation in defined intervals, the multiple surgical procedures themselves carry the risk of disease transmission (Young et al. 2005). To use tissue engineering with its gold standards for the repair of the traumatic lesions of bone and cartilage, two general approaches can be chosen from:

- The implantation of undifferentiated pluripotent stem cells (SCs) encapsulated in a scaffold;
- The implantation of tissue generated *in vitro*.

Using the first approach, undifferentiated cells are further differentiated towards a specific pathway in the desired environment. Regarding the second approach, tissue is generated using a bioreactor *in vitro* while undifferentiated pluripotent cells seeded in a scaffold host favour the formation of specific tissue prior to implantation (Lemoli et al. 2005).

2.1 STEM CELLS

SCs have the capacity to renew themselves and to produce specific cell types upon receiving signals. SCs categorize into two broad types and are generally termed as “totipotent or pluripotent” SCs since they have the potential to develop into many cell lineages. SCs can be:

- Embryonic Stem Cells (ESCs) (totipotent) or;
- Somatic Stem Cells (sSCs) (pluripotent)

ESCs are derived from the inner cell mass of the embryo or the epiblasts of the blastocysts and are capable of differentiating into the three germ layers: mesoderm, endoderm and ectoderm.

sSCs which include mesenchymal stem cells (MSCs), haematopoietic SCs, skin SCs, and adipose derived stromal cells, are termed as adult SCs. These types of cells are present in many tissues and have a high potential in differentiation along a specific lineage. Majority of adult SCs are totipotent, which means that they have limited differentiation capacity. For instance, haematopoietic SCs are multipotent or the skin’s SCs are

unipotent (Lemoli et al. 2005). Adult SCs can give rise to a colony of differentiated cells not identical to the original cell.

Apart from the differences of these two types of SCs, there exist minor issues that have stimulated research in the use of adult SCs instead of ESs. They are more accessible, and can be obtained in a larger quantity and their use has fewer ethical concerns (Lemoli et al. 2005).

2.2 BONE PHYSIOLOGY, BONE CELLS AND ECM

The musculoskeletal system is composed of bone, muscles, cartilage and tendons. Bone offers support, movement, protection, mineral storage (calcium, phosphorous, sodium, magnesium, and carbonate) and haematopoiesis (produces red blood cells, white blood cells and platelets). There exist two types of bone within the body; compact forming the external layer of bone and spongy forming the internal layer (Figure 2.1). Bone matrix is built up of 90% type I collagen and 10% of non-collagenous proteins as in osteocalcin, osteonectin, bone sialoprotein and various proteoglycans. These non-collagenous proteins are involved in the process of matrix maturation and mineralization as well as regulating the functional activity of the bone forming cells (Kassem et al. 2008). Bone consists of three types of cells which are as follows:

- Osteoblasts: Produces the organic part of the matrix osteoid which consists of proteoglycans, glycoproteins and collagen fibers. It is also involved in deposition of the inorganic components (mineral salts) as in HA.
- Osteocytes: Matured osteoblasts that are locked in the cavities of the bony matrix help the maintenance of its site. Osteocytes connected by Canaliculi

enable cellular communication through thin cytoplasmic extensions. Nutrients and oxygen passing through the blood vessels reach the cells by diffusion through the matrix.

- Osteoclasts: Giant multinucleated cells which are involved in resorption and remodelling of the bone.

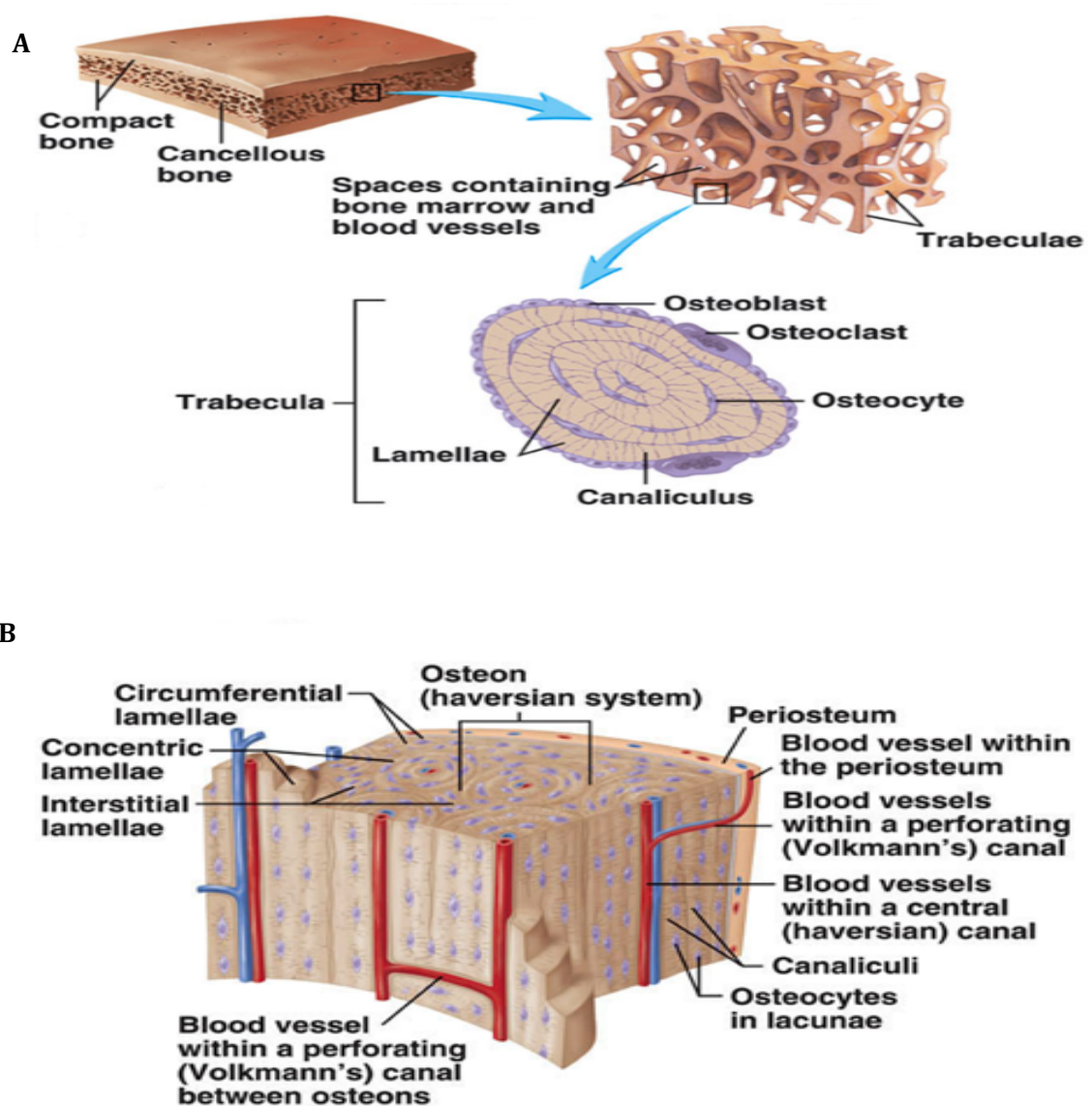


Fig. 2.1 Microscopic representation of (a) spongy bone (b) compact bone (The McGraw-Hill Companies, Inc.)

As Figure 2.2 shows, all bones consist of an outer surface called periosteum (except at the joints of long bones) as well as an inner part endosteum which lines along the bone marrow (BM) cavity. These connective tissues contain bone progenitor cells, which differentiate towards the osteoblastic lineage and secrete growth factors.

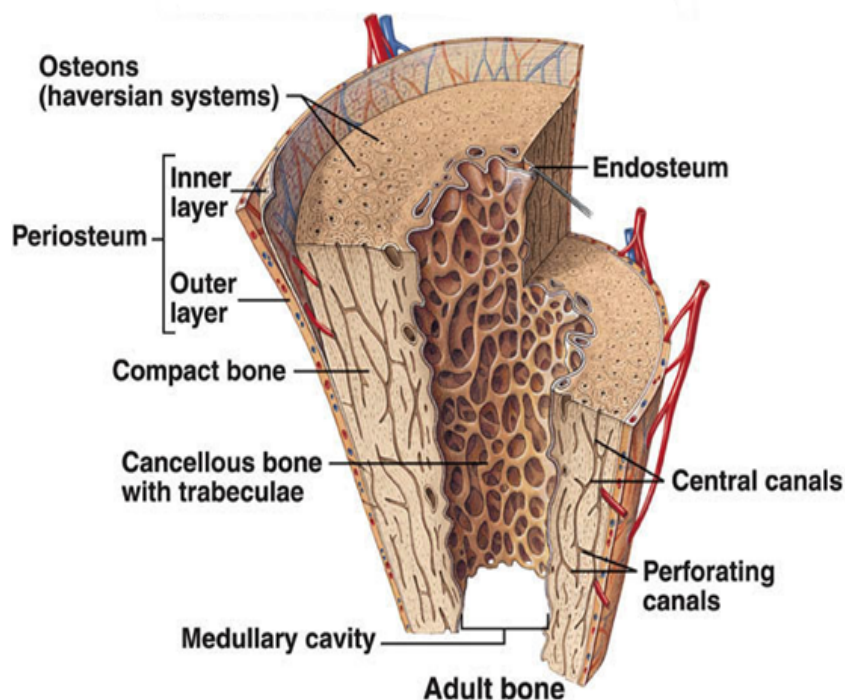


Fig. 2.2 Schematic representation of adult bone intersection (The McGraw-Hill Companies, Inc.)

Bone matrix is composed of the inorganic components (minerals) and the organic components (proteins). The bone mineral is mainly made of HA and the bone protein of triple helix collagen. Nanocrystals of HA are embedded to the collagen fibrils to provide strength and rigidity for the bone (Figure 2.3) (Murugan & Ramakrishna 2005).

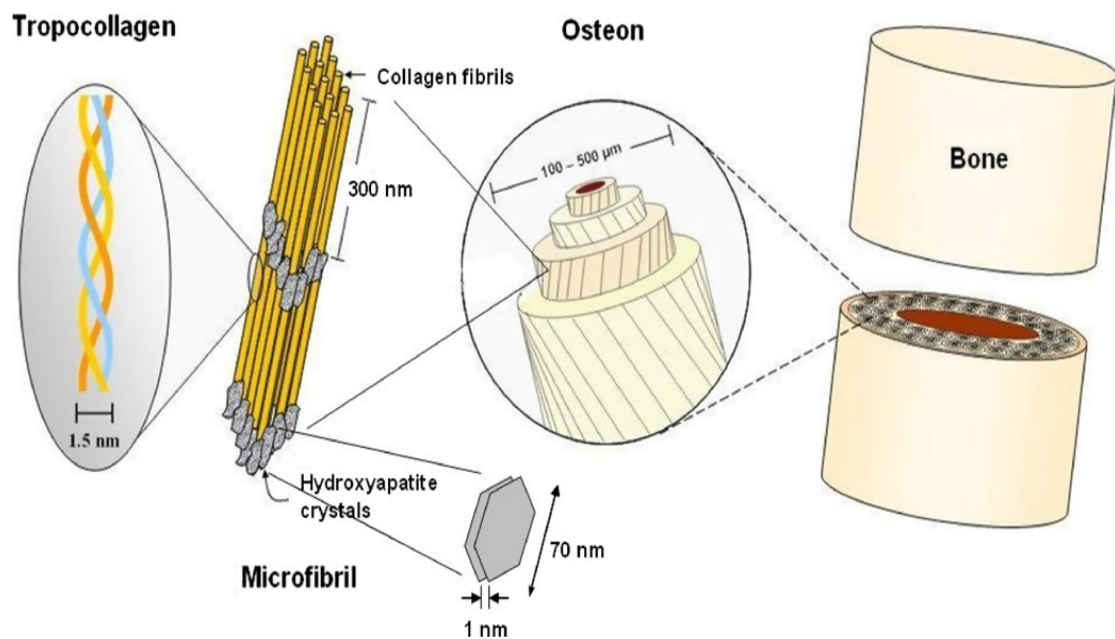


Fig. 2.3 Schematic representation of bone nanocomposite (Meyers et al. 2008)

Extracellular Matrix (ECM) is made up of large structural proteins, glycosaminoglycans and proteoglycans. It is the major constituent of multi-cellular organisms as it assists in cell signalling and tissue homeostasis as well as developmental processes and provides a supporting structure for cells and tissues (Kleinman et al. 2003). Therefore, the development and transformation of ECM is vital to tissue generation as it guides cells through migration and differentiation (Adams & Watt 1993). The ECM of each tissue amends its composition to the function of the specific tissue to enable interactions between matrix molecules, growth factors and cell surface receptors responsible for mediating adhesion of cells to the ECM (Cecilia 2010). For instance, collagen type I is the main constituent of bone ECM whereas collagen type II is mostly found in cartilage ECM.

2.3 TRAUMA AND TISSUE ENGINEERED BONE

Bone loss can be due to trauma, osteonecrosis, tumours and large bone defects (Braddock et al. 2001). Until today, autologous grafting (graft taken from the individual themselves) has been the best solution for the patients (Figure 2.4). However, this option has faced limitations of unavailability of sufficient donor tissue and even if available, difficulties in matching the size and quality of bone needed. Also such surgical procedures might cause donor-site morbidity. Tissue Engineering has addressed these limitations by regenerating bone through isolation of the individuals' own bone forming SCs for further encapsulation within a scaffold matrix while being nourished with nutrients and growth factors. This is done to tailor the specific bone graft needed for implantation (Braddock et al. 2001).

In order to reconstruct bone tissues, osteogenic SCs such as the MSCs can be isolated and induced to differentiate into osteoblasts (Triffitt 2002). Cells with chondro and osteogenic potential can be isolated from many tissues such as periosteum, BM, thymus, skeletal muscle and more. Friendenstein was the first who identified BM population of cells to have a strong osteogenic potential (Friednstein 1973). Due to their totipotency coupled with easy isolation from the tissue source, BM stromal cells are most frequently used in bone regeneration clinically. BM stromal cells can be differentiated to bone, cartilage, muscle, marrow stroma, tendon, fat and a number of other connective tissues upon stimulation (Lemoli et al. 2005). However, BMSCs are a heterogeneous population of mesenchymal progenitor cells and a prolonged time in culture decreases their multilineage potential. As a result, mechanical and biological

stimulus is needed to improve osteogenic differentiation potential (Matziolis et al. 2011).

One of the main limitations faced in the world of tissue regeneration is to find the most suitable scaffold with the right choice of cell type. This thesis addresses this limitation towards the use of BMSC seeded in biodegradable hydrogels.

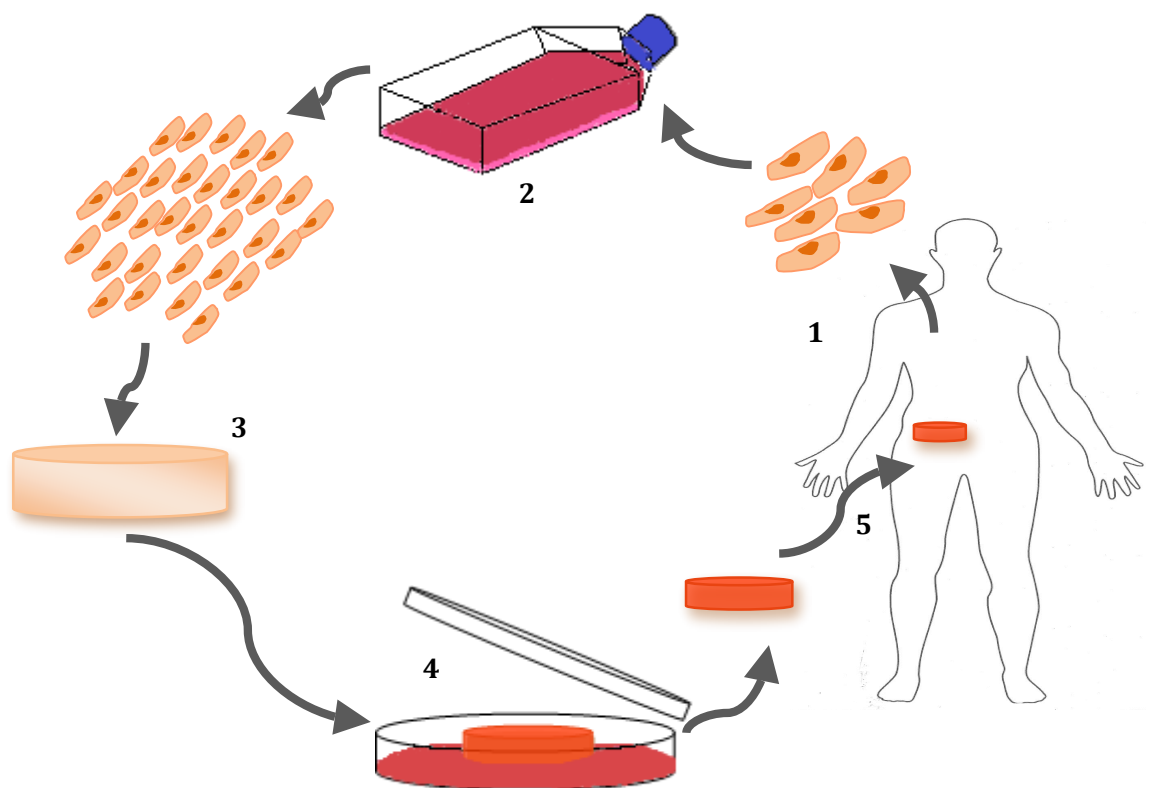


Fig. 2.1 Tissue engineering, using autologous grafting: (1) A number of cells are isolated from a tissue of the body, (2) screened for phenotype and expanded in culture, (3) cells are seeded onto porous scaffolds together with growth factors and nutrients (SDMEM) to enhance proliferation, (4) seeded scaffolds are incubated in culture media to further proliferate, (5) the scaffold containing the regenerated tissue is implanted into the defected area to integrate with the natural tissue.

2.4 DIFFERENTIATION OF MSCs ALONG OSTEOGENIC LINEAGE MSCs

MSCs are known as multipotent cells present in the marrow. These cells can be multiplied as undifferentiated cells and further differentiate into MSC lineages (bone, cartilage, fat, tendon, muscle and marrow stroma) (Pittenger et al. 1999). Differentiation of MSCs into osteoblasts requires the presence of β -glycerolphosphate, ascorbic acid and dexamethasone (Jaiswal et al. 1997; Kassem et al. 2008). When MSC monolayers are cultured in the presence of these supplements, they obtain an osteoblastic morphology with up-regulation of alkaline phosphatase (ALP) activity and deposition of a mineralized ECM. The mineralized matrix (Gkioni et al. 2010) can be detected (calcium deposits) via Alizarin Red assay or Von Kossa staining (Ogawa et al. 2004). There are also other assays to detect changes of cell surface expression of STRO-1 (Gronthos et al. 1994; Gronthos et al. 1999) and ALP antigen (Castano-Izquierdo et al. 2007; Giuseppe Maria de Peppo et al. 2010), the secreted osteocalcin (Jaiswal et al. 1997) or collagen type I (Wang et al. 2007). Other osteogenic markers such as the bone proteins (sialoprotein and osteopontin) could be detected using biochemical assays (Chevallier et al. 2010).

2.5 BONE GROWTH FACTORS FOR GROWTH/REPAIR

Bone has a regenerative potential to promote the formation and resorption of bone cells in the presence of specific growth factors such as IGFs, TGF β s and BMPs. Therefore, human bone matrix contains several growth factors which are grouped in Table 2.1 (Linkhart et al. 1996).

Table 2.1 Bone growth factors for growth/repair

Growth Factor	Symbol	Source/Function in bone formation
Insulin-like growth factors I, and II	IGF-I and IGF-II	<ul style="list-style-type: none"> Produced by bone cells, stored in bone matrix Stimulate osteoblastic cell proliferation and differentiation functions such as the expression of collagen I.
Transforming growth factors	TGF- β_1 and TGF- β_2	<ul style="list-style-type: none"> Increase osteoclast formation and is detected in osteoclasts.
Bone Morphogenic proteins	BMPs 2-7	<ul style="list-style-type: none"> Induce cartilage and bone formation from non-skeletal mesodermal cells. Makes bone through enchondral ossification.

3. BIOPOLYMERS, RHEOLOGY, COMPRESSION AND BIOCERAMICS

Principally, cell seeded hydrogels are prepared through mixing of the hydrogel solutions with cells, followed by gelation through either physical or chemical cross-linking methods. A number of factors control SC differentiation such as growth factors, matrices and forces (Discher et al. 2009). The hydrogel constructs must possess sufficient mechanical stiffness to maintain their structures against different mechanical forces in a bioreactor or at an implant site (Clark 1991). Moreover, SCs direct their differentiation towards a specific lineage when cultured on substrates matching the stiffness corresponding to the specific native tissue. For instance, neural SCs depending on the matrix elasticity differentiates into the neural or glial lineage (Saha et al. 2008), and multipotent MSCs (Pittenger et al. 1999) get directed towards either neurogenic lineage, myogenic or osteogenic lineage once cultured on substrates having neural, muscle or bone substrate stiffness, respectively (Engler et al. 2006a;Rowlands et al. 2008;Tse & Engler 2011). Moreover, it has been reported that stiffness of a scaffold is an effective regulator of SC differentiation as well as affecting the cell migration (Breuls et al. 2008;Tse & Engler 2011). As a result, many studies have been undertaken on the degradation and rheological properties of biopolymers (Chen et al. 2002; Hunt et al. 2007;Bott et al. 2010).

3.1 SCAFFOLDS USED IN TISSUE ENGINEERING

An ideal scaffold used in tissue engineering should exhibit characteristics similar to the natural ECM deposited in the body. The main features are as follows: (Freed et al. 1994;Lemoli et al. 2005)

- High surface area for cell-polymer interactions;
- Sufficient space for extracellular matrix regeneration;
- Easy diffusion of nutrients and cellular waste products during *in vitro* culture;
- Resorb once it has provided a template for regeneration of tissue;
- Degradation rate should be adjustable to match the rate of tissue regeneration;
- Provide good initial mechanical support for cells and ECM during the repair process;
- Non-toxic and non-immunogenic;
- Low cost of manufacture.

Biopolymeric scaffolds have been commonly used in regenerative medicine as the chemical composition of their monomer units can be flexibly tailored during synthesis. They may be modified with adsorbed proteins or immobilized functional groups (Gkioni et al. 2010; Glowacki & Mizuno 2008).

3.2 GELATION MECHANISM OF POLYMER GELS

Around 70 years ago, Flory (1941) and Stockmayer (1943) first introduced the theory of polymer gelation (Flory 1941b; Stockmayer 1943). In principal, when the intermolecular bonds within the polymer solution dominate over the intramolecular forces, crosslinking and branching takes place which will lead to gelation (transition from liquid to soft solids) (Ross-Murphy et al. 1998). The gel point is the critical point at which transition from liquid to soft solids takes place. At this point, the steady shear viscosity is infinite and its equilibrium modulus zero (Winter & Chambon 1986). Flory proposed an equation in the year 1941 which states that there are 'f' potential binding

sites in each polymer chain, of which a minimum fraction, α_c , must react for a three-dimensional network to form. The equation 3.1 showing this correlation is as follows (Flory 1941a):

$$\alpha_c = 1/(f - 1) \quad \text{Equation 3.1}$$

As the degree of conversion reaches α_c , viscosity increases sharply, molecular weight (MW) raises to infinity and a continuous network of polymer chains form (Ross-Murphy et al. 1998).

3.3 HYDROGELS

Hydrogels are hydrophilic polymers crosslinked through the association of hydrogen bonds or van der Waals interactions between the chains. As hydrogels come in contact with water, they swell forming an insoluble 3D network. This is beneficial to the cells as it protects them while they are embedded within the gel network for a controlled delivery to the injury site. The aqueous matrix of gels facilitates the transportation of nutrients and cell metabolism by-products in and out of the gel. Table 3.1 outlines the classification of present hydrogels (Gkioni et al. 2010).

Table 3.1 Biopolymer Gels

Origin		Biopolymer Gel	
Natural	➤ Anionic	<ul style="list-style-type: none"> • Hyaluronic acid • Alginate • Pectin 	<ul style="list-style-type: none"> • Carrageenan • Chondroitin Sulphate • Dextran Sulphate
	➤ Cationic	<ul style="list-style-type: none"> • Chitosan 	<ul style="list-style-type: none"> • Polylysine
	➤ Amphipathic	<ul style="list-style-type: none"> • Collagen (and gelatin) • Fibrin 	<ul style="list-style-type: none"> • Carboxymethyl chitin
	➤ Neutral	<ul style="list-style-type: none"> • Dextran • Agarose 	<ul style="list-style-type: none"> • Pollulan
Synthetic	➤ Polyesters	<ul style="list-style-type: none"> • PEG-PLA-PEG • PEG-PLGA-PEG • PEG-PCL-PEG • P(PEG/PBO terephthalate) 	<ul style="list-style-type: none"> • PLA-PEG-PLA • PHB • P(PF-co-EG) ± acrylate end groups
	➤ Others	<ul style="list-style-type: none"> • PEG-bis-(PLA-acrylate) • PEG±CDs • PEG-g-P(AAm-co-Vamine) • PAAm • P(NIPAAm-co-AAc) • P(NIPAAm-co-EMA) 	<ul style="list-style-type: none"> • PVAc/PVA • PNVP • P(MMA-co-HEMA) • P(NA-co-allyl sulphate) • P(biscarboxy-phenoxy-phosphazene) • P(GEMA-sulphate)
Natural + Synthetic		<ul style="list-style-type: none"> • P(PEG-co-peptides) • P(PLGA-co-serine) • P(HPMA-g-peptide) • P(HEMA/Matrigel) 	<ul style="list-style-type: none"> • Alginate-g-(PEO-PPO-PEO) • Collagen-acrylate • Alginate-acrylate • Hyaluronic-g-NIPAAm

Hydrogels are crosslinked in a number of ways chemically as well as physically (Hennink & van Nostrum 2002), Table 3.2 outlines a number of methods used.

Table 3.2 Methods of chemically and physically crosslinking hydrogels

Hydrogels Chemically Crosslinked with:
<ul style="list-style-type: none"> • Radical Polymerization • High energy irradiation • Chemical reaction of complementary groups (with aldehyde, addition reactions and by condensation reactions) • Using Enzymes
Hydrogels Physically Crosslinked with:
<ul style="list-style-type: none"> • Ionic interactions • From amphiphilic blocks and graft copolymers (block and graft copolymers of PEG and PL(G)A); Hydrophobized polysaccharides; other block and graft copolymers • Crystallization (in homopolymer systems or by sterocomplex formation) • Hydrogen bonds • Protein interactions (use of genetically engineered protein; antigen antibody interactions)

3.3.1 ALGINATE

Alginates are polysaccharides derived from brown algae and bacteria. Chemically, they are linear copolymers composed of 1→4 glycosidically linked β -D-mannuronate (M residues) and 1, 4-linked α -L-guluronate (G residues) (Figure 3.1). The residues are present as units of each monomer and are separated by regions in which they are randomly arranged or alternating with MG blocks. Blocks can be in the form of homopolymeric M-units (M-M-M) (Figure 3.2), homopolymeric G-units (G-G-G) (Figure

3.3) and heteropolymeric MG-units (G-M-G-M). Guluronate blocks are mainly responsible for the formation of calcium crosslinked hydrogels (Draget & Taylor 2011). The proportions of (M) and (G) residues and lengths can vary considerably depending on the source of the alginate. However, it has been reported that C-5 epimerases enable the tuning of alginate structure (Donati et al. 2005). A high content of guluronic acid blocks can result in a higher strength gel compared to alginates rich in mannuronate blocks (Draget et al. 1994). A recent study has reported that a certain fraction of G-M blocks when formed with high MW alginate gives a negative immunogenic response *in vivo* (Draget & Taylor 2011). This response had been reported and explained by Stokke et al. (1993), to be due to the leaching out of the mannuronate-rich fragments that do not take part in the gel network which is observed in the overgrowth of the capsules (Stokke et al. 1993). It is believed that this could be partly-linked to the β -(1 \rightarrow 4) glycosidic linkages since other homopolymeric di-equatorial polyuronates, like D-glucuronic acid also exhibit this feature (Skjak-Braek & T.Espevik 1996). They believe that this triggers an immune response.

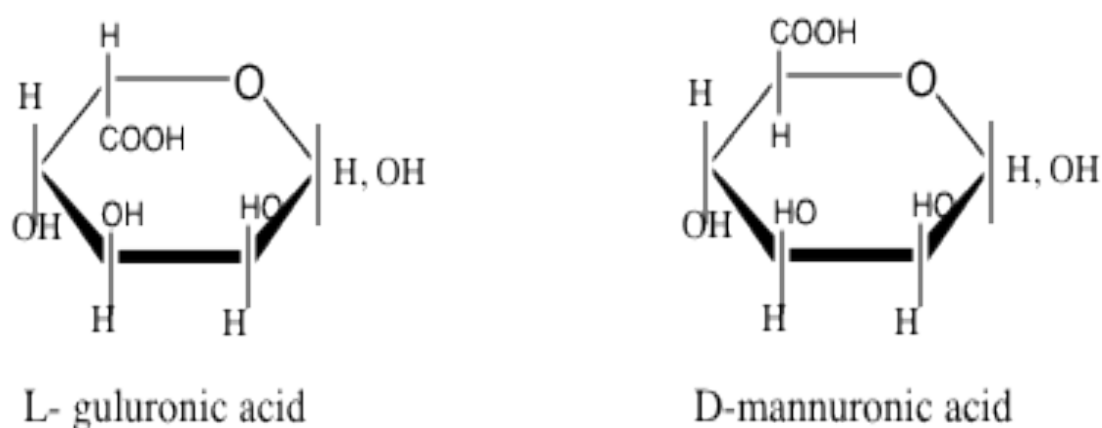
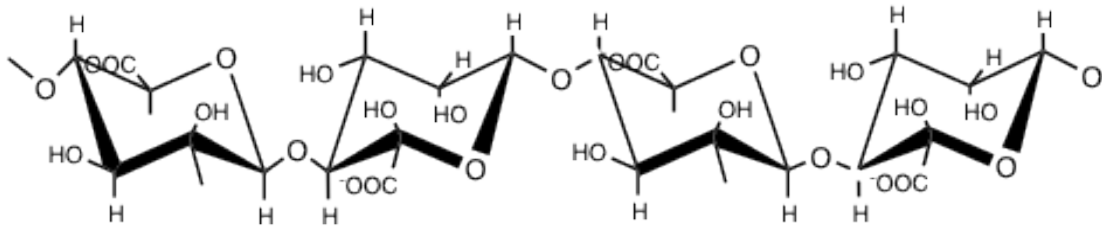


Fig. 3.1 L-guluronic acid and D-mannuronic acid monomers

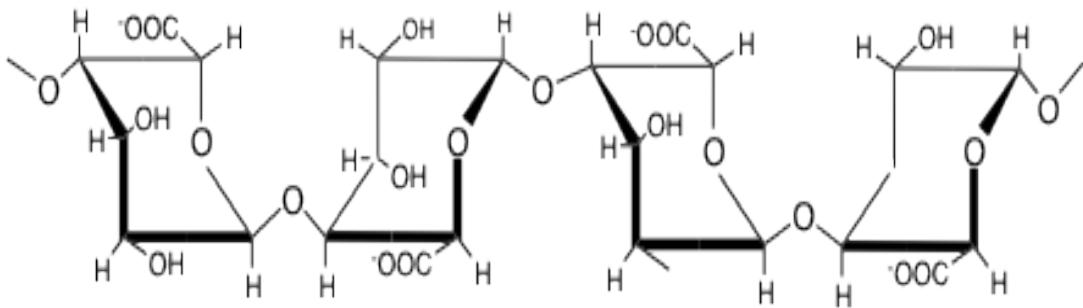
MM Units



Joined with $\beta(1 \rightarrow 4)$ bonds D-mannuronic acid

Fig. 3.2 Mannuronic units (MM) of alginate (Adapted from Khotimchenko et al. 2001)

GG Units



Joined with $\alpha(1 \rightarrow 4)$ bonds L-guluronic acid

Fig. 3.3 Guluronic unit (GG) of alginate (Adapted from Khotimchenko et al. 2001)

Alginate is produced through a postpolymerization reaction that involves inversion of a C-5 on the M residues of mannuronan. It undergoes ionotropic gelation in the presence of divalent and polyvalent cations in particular calcium ions (Kohn 1975). The structure of alginate gel is formed through the interaction of 'egg-box' junctions (Figure 3.4). The model proposes that the egg-box dimers are formed through a pair of buckled G sequences (G blocks have a 2/1 helical formation forming buckled regions) with the incorporation of the divalent cations such as Ca^{2+} within the cavities (Fang et al. 2008). The binding of Ca^{2+} takes place in three steps: (I) viscosity reduces as the

individual alginate chains collapse/shrink. Monocomplexes formed between Ca^{2+} and G blocks. The reduction in the viscosity results from a decrease/increase of the intermolecular repulsion/attraction forces. Step II involves the growth of molecular size and formation of egg-box dimers by pairing of the monocomplexes. This step is thus strongly exothermic. In step III, the egg-box dimers are laterally associated to build multimers (Fang et al. 2008).

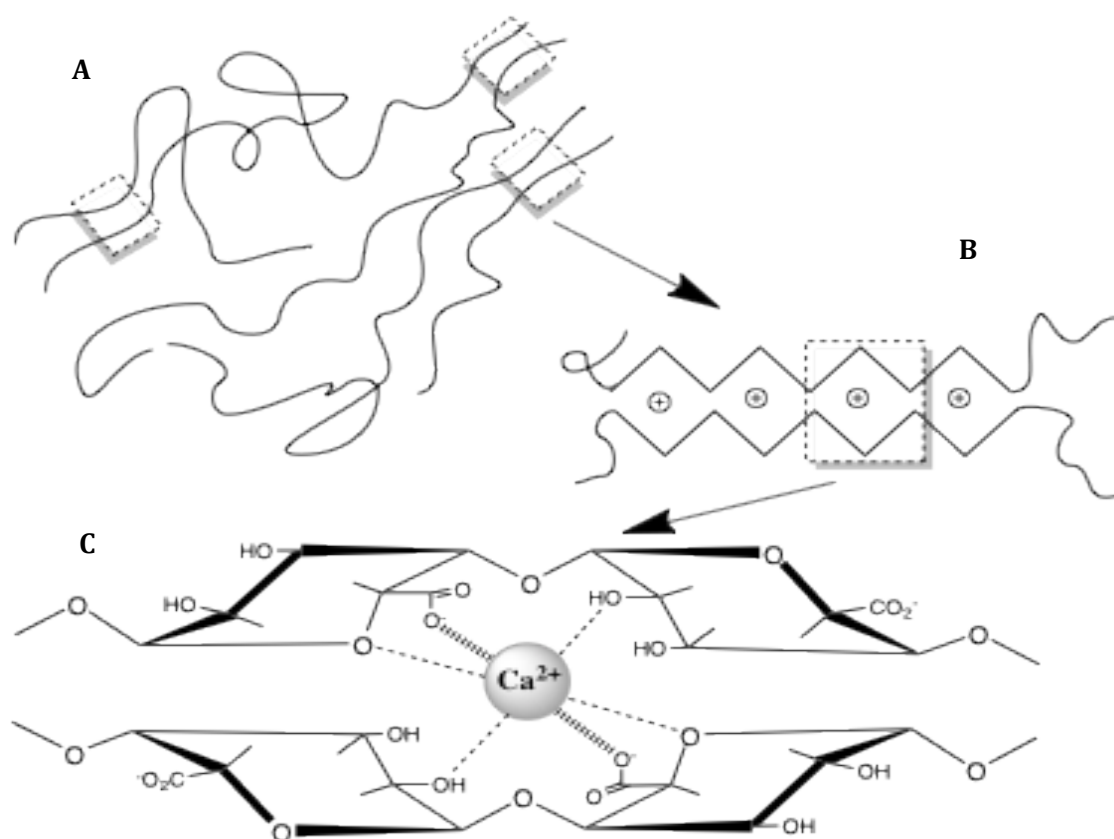


Fig. 3.4 Gelation process of alginate crosslinked by Ca^{2+} ; eggbox model; (a) strands of alginate; (b) coordination of Ca^{2+} in a cavity formed by a pair of gluturonate units along alginate chains; (c) egg-box dimer, dotted line indicates hydrogen bonds between oxygen atoms of the pyranosic cycles and Ca^{2+} ; dashed lines indicates ionic bonds between carboxyl groups and Ca^{2+} . (Modified from McHugh et al. 1987)

It is reported that physical crosslinking by ionic interactions instead of chemical crosslinking reduces the chances of toxicity of the reagents (Hou et al. 2008) which is important in cell encapsulation. The strength of alginate gel is positively correlated with the concentration of the type of ion (calcium ion) and alginate solution (Hou et al. 2008), thus it could be easily adjusted to the purpose. The gelation temperature of this polymer increases with polymer concentration (Florian-Algarin & Acevedo-Rullan 2008). This polysaccharide is widely used in tissue regeneration and biotechnology as it has high water content and supports high oxygen permeability. Studies show that cells remain viable in alginate-encapsulated form for a significant period of time (Grover & Smith, 2009; Hunt et al. 2010). The viscosity of the pre-gelled solution is important in maintaining cell viability. High viscosity is not favourable during the pre-gel cell mixing process since high shear forces exerted on the sensitive cell membranes might lead to cell death. Therefore, mechanically rigid alginate hydrogels with high number of viable encapsulated cells should be prepared from solutions of low viscosity. In this context, a study done by Kong, Smith and Mooney in the year 2003, reported that alginate molecules could be modified to have a lower MW while maintaining their gel-forming ability. This was done through selective chain breakages in the component of the polymer chains that do not take part in the gel formation. This was done while high elastic modulus of the hydrogel was still achieved (Kong et al. 2003).

Sodium alginate gel possesses a relatively good mechanical stability and at the same time is physically non-permanent around room temperature as degradation is important in tissue engineering applications. Calcium alginate gel is dissolved *in vivo* by the action of enzymolysis, and it is reported that the products are non-harmful to

the normal tissues (Heald et al. 1994;Ueng et al. 2000). Alginate gel is well characterized and is amendable. It can be chemically modified and is easily sterilized (Ratner et al. 1996). Alginates are water soluble when bound to monovalent ions, whereas hydrogels which are partially soluble in water are formed when alginate binds to divalent cations like Ca^{2+} (Tampieri et al. 2005). Alginate can be gelled externally and internally. External gelation takes place when soluble CaCl_2 is added to alginate to reach an instantaneous gelation. Internal gelation however takes place when solid CaCO_3 is added to the alginate solution, Ca^{2+} cations are released gradually through the slow hydrolysis of D-Glucone- δ -lactone (GDL). The G-blocks crosslink as a result of adding Ca^{2+} ions from CaCO_3 , forming a more packed 'crosslinked' which is known as an egg-box formation. Manipulation of the MW distribution (M:G ratio), crosslinking cation density, alginate concentration and gelation temperature influences the mechanical properties of alginate (Kuo & Ma 2001).

One important limitation in using alginate gels in tissue engineering is the lack of cellular interaction. Its hydrophilic nature does not allow adsorption of proteins and thus is unable to interact with mammalian cells. As a result of this limitation, alginate is modified with lectin, that is a carbohydrate specific binding protein, as well as covalently being bonded to an RGD-containing cell adhesion ligand to improve cell adhesion (Lee & Mooney 2001;Rowley et al. 1999;Sultzbaugh & Speaker 1996).

3.3.2 GELLAN GUM

Gellan gum is a microbial exopolysaccharide produced by fermentation of the organism *Pseudomonas elodea* (Dai et al. 2008; Doner 1997). The structure of

deacylated gellan gum polymer consists of linear anionic tetrasaccharide repeating units of two glucoses, rhamnose and glucuronic acid residues: ($\rightarrow 3$)- β -D-Glcp-($1 \rightarrow 4$)- β -D-GlcpA-($1 \rightarrow 4$)- β -D-Glcp-($1 \rightarrow 4$)- α -L-Rhap-($1 \rightarrow$) (Figure 3.5) (Jansson et al. 1983).

Gellan appears in two forms, native gellan which is called high acyl (Tako et al. 2009) and commercial low-acyl gellan. In high acyl gellan, the first glucose residue undergoes a substitution of L-glycerate group at C2 and an acetate group at C6 at about 50% replacement level (Kuo et al. 1986). The three oxygen atoms present in the glycerate group stabilizes the double helix structure of gellan by forming four new interchain hydrogen and ion bonding (Chandrasekaran et al. 1988;Huang et al. 2003). Removing the acetate and glycerate groups using a strong alkali treatment produces the low-acyl gellan. High acyl gels require higher temperature to form gels and are essentially more stable in comparison to low acyl gellan. This is why high acyl gellan forms softer gels than the low acyl gellan (Morris et al. 1996).

Gellan gum forms a 3D triangular 3-fold double helix via two left handed chains coiled around one another with the acetate residues on the periphery and glyceryl groups stabilizing the interchain associations. Hydrogen bonds are formed between the hydroxy methyl group of 4-linked glucosyl units of one chain and the carboxylate group of the other. In order to promote gelation, divalent cations (preferably divalent) as in Ca^{2+} and Mg^{2+} are introduced to the ion-binding sites, the chain then undergoes a conformational transition from random coils to double helices and the rearrangements of the double helices leads to the formation of ordered junction zones, ultimately leading to pairs of helices (Matricardi et al. 2009). In the case of the addition of monovalent cations as in Na^+ and K^+ , these ions suppress the repulsion by binding to

the surface of the helices balancing the negative charge of the carboxyl groups and require to be present in higher concentration to promote gelation (Smith et al. 2007). The three-dimensional hydrophilic polymeric network that gellan forms contain great water absorbance capacity which highly resembles biological tissues and thus, is widely used in biomedical applications (Matricardi et al. 2009).

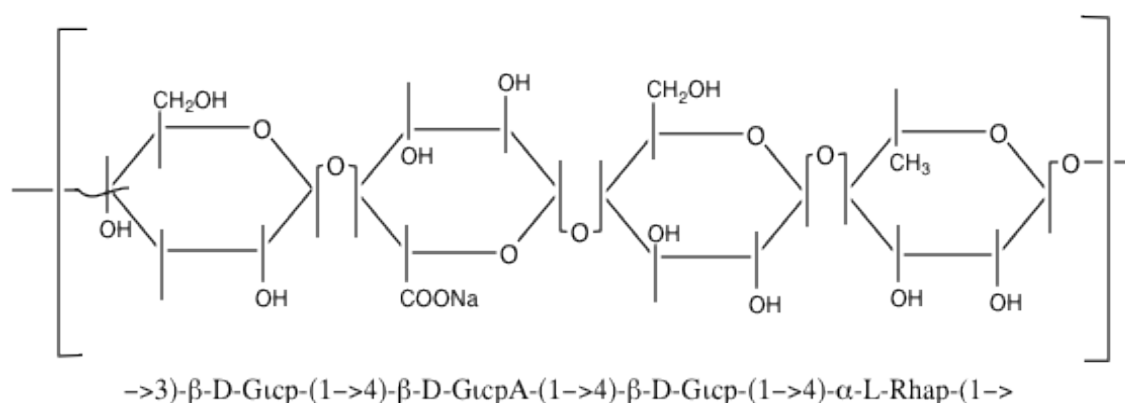
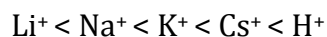


Figure 3.5 Chemical Structure of gellan gum repeating units reading from left: D-glucose, D-glucose, L-rhamnose and D-glucuronic acid.

Due to the thermoreversible nature of this hydrogel, the aqueous gellan gum solution containing coiled gellan chains dispersed in solution transfers into hydrogels with cooling. This is when the side-by-side chains wind around the closest point forming a discontinuous double helix structure. The cations added to further stiffen the gel gather around the helix part (Figure 3.6) (Dai et al. 2008).

Gellan gum hydrogel texture can be controlled by the added salts (Ogawa 1996). Thus, the gelation temperature and gel strength is highly dependent on the polymer concentration and cation species (Dai et al. 2008). A study undertaken (1987) by Grasdalen and Smidsrod categorised the monovalent and divalent cations in order of increasing gellan gum's gel strength (Grasdalen & Smidsrod 1987). They reported that

for monovalent cations at ionic strength of 0.1 M, the gel strength increases in the order of:



And for divalent cations:

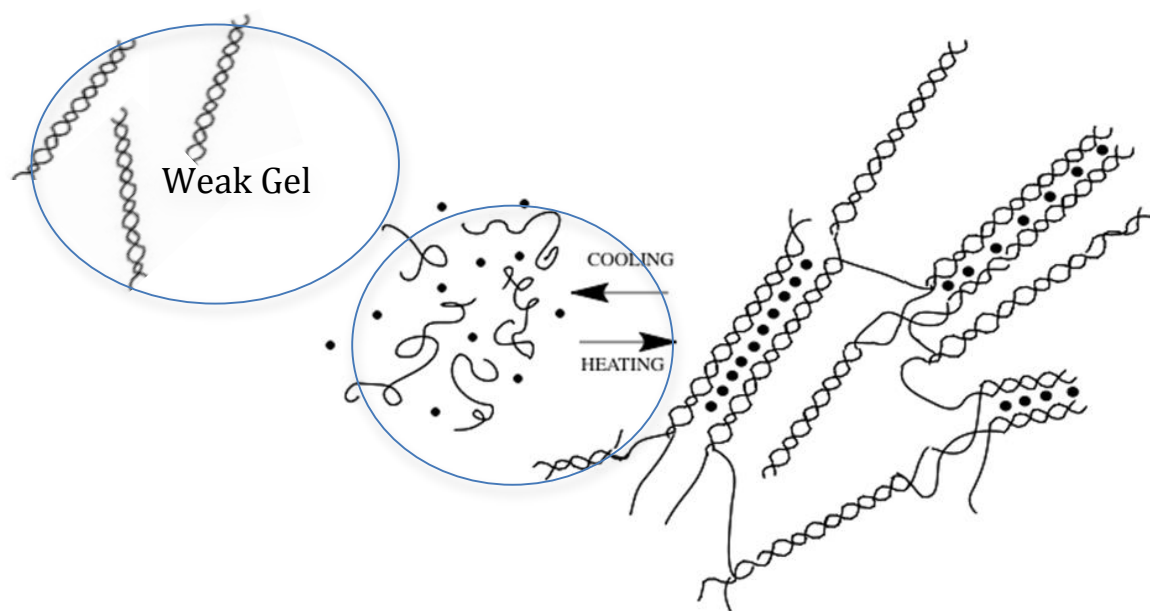
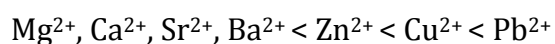


Fig. 3.6 Gellation mechanism of Gellan gum (double helix structure); ● denotes cations such as Ca^{2+} ions. (Adapted & modified from Hember et al. 1994)

3.3.3 PECTIN

Pectins are complex polysaccharides containing 1,4-linked α -D-galactosyluronic residues. The three pectic polysaccharides that are homogalacturonan, rhamnogalacturonan-I and substituted galacturonans can be isolated from primary

plant cell walls. Homogalacturonan (HG) is a linear chain of 1,4-linked α -D-galactosyluronic residues, in which some of the carboxyl groups are methyl esterified.

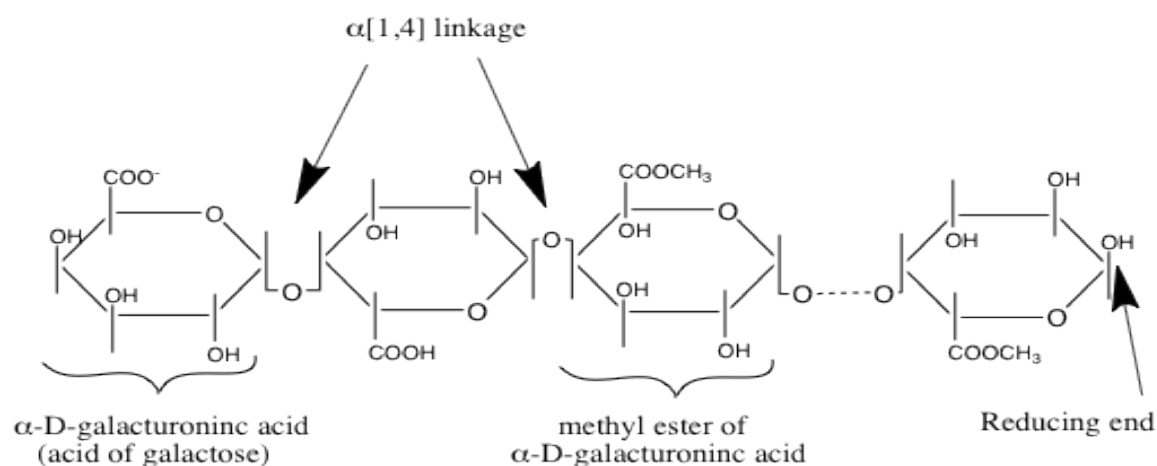


Fig 3.7 Chemical Structure of pectin repeating units

LM pectins can gel in the presence of divalent cations, commonly calcium ions. In these systems, gelation takes place by the formation of intermolecular junction zones between homogalacturonic smooth regions of different chains. The structure of such a junction zone is generally ascribed to the egg-box binding process. Initial strong association of two polymers into a dimer is followed by the formation of weak interdimer aggregation that is mainly governed by electrostatic interactions. The gel forming ability of LM pectins increases with decreasing the degree of methylation. LM pectins with a block-wise distribution of free carboxyl groups are very sensitive to low calcium levels. The presence of acetyl groups prevent the gel formation with calcium ions but gives the pectin emulsion stabilizing properties (Sharma & Naresh 2006).

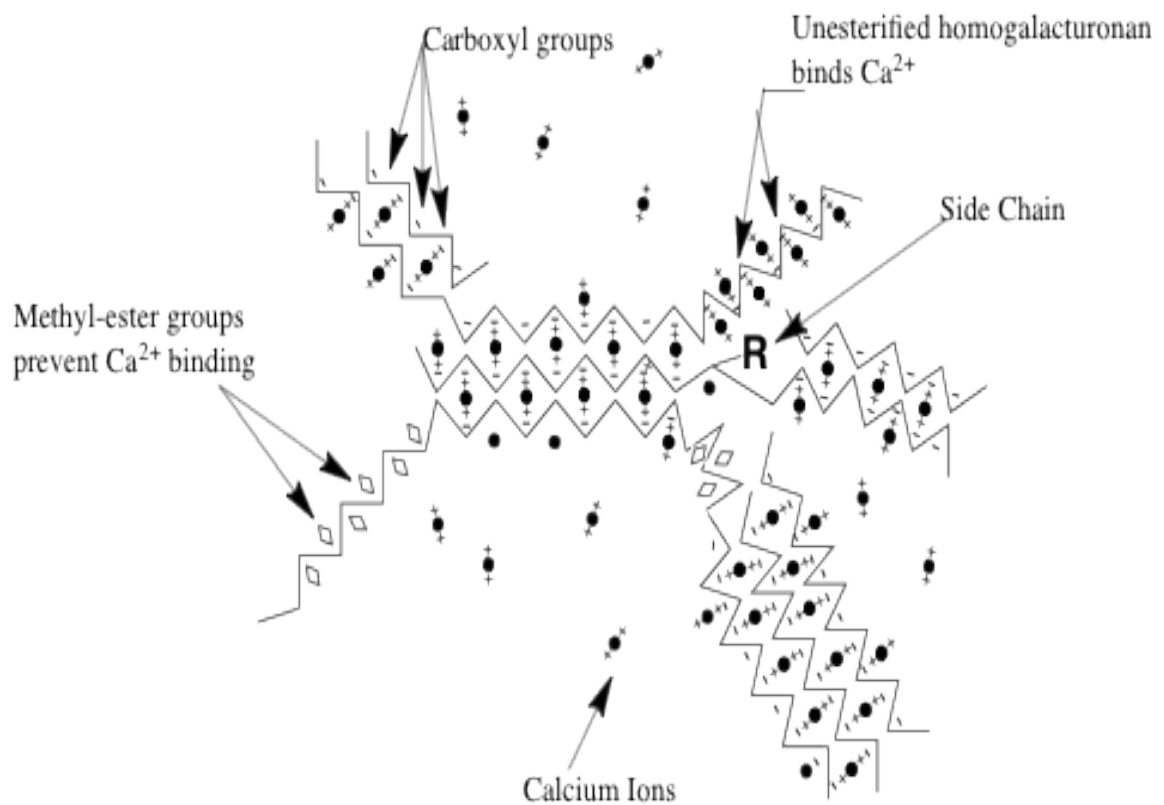


Fig 3.8 Egg-box model; representing the laterally associated egg-box multimers in gelation of LM pectin. (Adapted from Taiz & Zeiger 1998)

3.3.4 OTHER HYDROGELS

3.3.4.1 FIBRIN GEL: Is a natural polymer produced during blood coagulation. It plays an important role in haemostasis and natural wound healing (Albala & Lawson 2006; Janmey et al. 2009). Fibrin is used as sealant and adhesive in surgery (Ye et al. 2000). It forms gels through enzymatic polymerization of fibrinogen in the presence of thrombin (Janmey et al. 2009). Fibrin naturally forms a scaffold around trauma through building a three dimensional network of branching fibers. The structure and mechanical property of fibrin gels (plasma proteins) are similar to those of blood clot (Janmey et al. 2009). Fibrin can be used as an autologous scaffold as it can be provided from the

patients' blood, therefore will neither show any inflammatory responses (Ye et al. 2000) nor produces any toxic products following degradation (Ye et al. 2000). It can be crosslinked with the additives such as factor XIII (Karp et al. 2004). Fibrin is also used as a scaffold in cardiovascular tissue engineering (Jockenhoevel et al. 2001) and cell delivery systems (Bensaid et al. 2003;Karp et al. 2004). In the cell delivery system fibrin is used for the purpose of filling in the bone cavities and stopping the after bleedings in the final stage of coagulation to treat bone infections. Fibrinogen molecules are cleaved by thrombin, converted into fibrin monomers and assembled into fibrils where 3D networks of fibres are formed. Fibrin gel is osteoconductive and has homeostatic effect, also is biodegradable and porous which makes it suitable for use as a scaffold in bone tissue engineering (Bensaid et al. 2003;Hou et al. 2008). Fibrin can be used as a scaffold in cartilage tissue engineering; however, it degrades rapidly but excessive degradation can be controllable with the use of specific amounts of calcium and thrombin (Eyrich et al. 2007). It is also reported that the rate of degradation and remodelling by cell-associated enzymatic activity during cell migration and wound healing can be controlled with the use of aprotinin and proteinase inhibitors (Lee & Mooney 2001). Yet, the limited mechanical strength of this gel might cause limitations in its use in some tissue engineering applications (Lee & Mooney 2001).

3.3.4.2 COLLAGEN: Is a tissue-derived natural polymer making around 20-30% of total body protein (collagen, hyaluronan, and glycosaminoglycans) (Glowacki & Mizuno 2008;Lee et al. 2001a). It is found in the extracellular matrix of skin, bone, cartilage, tendon and ligament tissues (Lee & Mooney 2001). The biocompatibility, biological properties and weak antigenicity of this abundant polymer have enhanced its use in the

field of biotechnology (Lee et al. 2001a). Collagen can be used in native form or as denatured gelatin. There are at least 19 different types of collagen in the body (Lee et al. 2001a), Type I collagen is most commonly used. Collagen is made of three polypeptide chains wrapped around each other forming a three-stranded rope structure held together by both covalent and hydrogen bonds. They have the ability to self-aggregate to form stable fibers (Drury et al. 2004; Lee et al. 2001a; Yamamoto et al. 2003). Mechanical crosslinkers as in glutaraldehyde, formaldehyde and carbodiimide can chemically form collagen fibers and scaffolds (Lee et al. 2001b; Park et al. 2002). To physically form gels, UV irradiation, freeze-drying and heating can be applied (Lee et al. 2001b; Schoof et al. 2001). It naturally degrades by metalloproteases (collagenase) and serine proteases and further allows the cells present in engineered tissue to locally control its degradation (Drury et al. 2004). Ubiquitous type I collagen derived from bovine which allows good cell adhesion is widely used as a scaffold for tissue repair (Badylak 2002). The limitation of collagen gel is its mechanical weakness, which reinforcement with solid components and alignment during gelation and culture could improve its performance (Wallace & Rosenblatt 2003). This gel can be useful on its own, in combinations or with modifications in regenerative medicine.

3.3.4.3 GELATIN: Is formed by breaking the triple-helix structure of collagen into single stranded molecules (Lee & Mooney 2001). There are two types of gelatin type A and B which the former is prepared by acidic treatment before thermal denaturation and the latter is produced by alkaline treatment leading to high carboxylic content (Lee & Mooney 2001). Gelatin is widely used in medical and pharmaceutical applications (Kuijpers et al. 2000; Yamamoto et al. 2003; Young et al. 2005). Some of the drawbacks in

gelatin based applications for tissue engineering is the weakness of the gels formed which can be improved (Kuijpers et al. 2000; Lee & Mooney 2001).

3.3.4.4 CARRAGEENAN: Is from the family of sulphated polysaccharides extracted from red seaweeds. It exists in 3 forms of kappa, lamda and iota that differ in the number and position of the ester sulphate groups on the galactose unit. It is a high MW polysaccharide made up of repeated units of galactose and 3,6 anhydrogalactose (3,6-AG) which are joined by alternating alpha 1-3 and beta 1-4 glycosidic linkages. Kappa Carageenan form gel in the presence of hydrophobic cations, such as potassium, rubidium, cesium and sodium (MacArtain et al. 2003). Its gelling mechanism is similar to the gelation mechanism of gellan gum. Many studies have looked at the rheological measurements of carrageenan (Eleya & Turgeon 2000). A recent study by Thrimawithana (2010) has investigated the texture and rheological characterization of kappa and iota carrageenan in the presence of counter ions. Studies have reported that as carrageenan degrades *in vivo*, it could cause ulcerations in the gastrointestinal tract and cause cancer (Tobacman 2001). Thus its use in tissue engineering is limited.

3.3.4.5 AGAROSE: Is a type of marine algal polysaccharide that forms thermally reversible gels. It forms bundles of associated double helices upon gelation, and its junction zones consist of multiple chain aggregation. Agarose physical structure as in pore sizes can easily be tailored by using different concentrations of the gel. Low concentration of agarose results in large pore size and low mechanical stiffness which is beneficial for migration and proliferation of the cells. However, since the gel is non-degradable, it is not widely used in regenerative medicine (Lee & Mooney 2001).

Other hydrogels with biological applications include Hyaluronate, dextran and Chitosan.

3.4 BIOPOLYMER RHEOLOGY

Biopolymer rheology is a method used to determine and monitor the rheological properties of the final gels. A general definition of rheology is the measure of flow and deformation of materials (Barnes H.A. et al. 1989). It can also be defined as the relationship between stress, σ , (force per unit area, Pascal) (Equation 3.2) and strain, γ , (resulting fractional deformation, dimensionless ratio) within the material as a function of time. The word itself originates from Ancient Greek language; “logy” is the study of “rheo” which is flow. The extreme states that materials can exist in are solids (behaving as a perfect Hookean) and liquids (behaving as a perfect Newtonian). In solids, σ is proportional to γ and independent of the rate of γ . In liquids, σ is proportional to the rate of γ but independent of γ itself (Chhabra 2010). Viscous biopolymer gels belong to the mid-extreme of the two states, called viscoelastic which means that the behaviour of the gels is elastically both solid and liquid. In the case of biopolymers where the samples exist in a state of soft solid, shear force (F) (deformation) is applied laterally upon the sample area (A) to measure the stress (σ).

$$\sigma = F/A \quad \text{Equation 3.2}$$

Shear modulus (G) is calculated from stress over strain (Equation 3.3).

$$G = \sigma/\gamma \quad \text{Equation 3.3}$$

$\tan \delta$ is the loss tangent which is derived from the loss and storage modulus (Equation 3.4)

$$\tan \delta = G''/G' \quad \text{Equation 3.4}$$

Small deformation oscillatory rheometer sinusoidal strain wave is applied on the hydrogel samples to measure the mechanical properties of the gels. Strain wave with fixed amplitude of (γ_0) and frequency of strain wave (ω) is applied. The resulting shear stress (σ_0) transmitted through the sample can be resolved into its viscous and elastic components that can be resolved from the in phase and out of phase response.

The elastic components defined as the storage modulus (G') is the ratio of in-phase stress (σ_0) to the strain (γ_0) (Equation 3.5).

$$G' = \sigma_0 / \gamma_0 \cos \delta \quad \text{Equation 3.5}$$

The viscous components defined as the loss modulus (G'') is the ratio of out of phase stress (σ_0) to the strain (γ_0) (Equation 3.6).

$$G'' = \sigma_0 / \gamma_0 \sin \delta \quad \text{Equation 3.6}$$

Complex dynamic viscosity (η^*) is calculated through the following equation where the frequency of the strain wave (ω) is the oscillatory analogue of shear rate given by $2\pi \times$ frequency (Hertz) (Equation 3.7).

$$\eta^* = \sqrt{(G')^2 + (G'')^2} / \omega \quad \text{Equation 3.7}$$

These moduli are measured over a range of frequencies to produce a plot representing the mechanical spectra of the samples providing a profile of the characteristic structural properties.

The process of gelation takes initiates with a solution of dilute polysaccharide with liquid like characteristics which represent a spectra of $G'' > G'$. Both moduli increase with frequency proportional to ω and ω^2 and η^* independent of ω . With increase in the concentration of the polymer, chains interpenetrate resulting in entanglement where $G'' > G'$, η^* independent of ω . Then the polymer chains rearrange within the period of oscillation. With an increase in the frequency of oscillation, an elastic response is obtained with $G' > G''$, η^* decreases sharply with ω . At high frequency, the disentangled chains become more associated resulting in a gel like spectrum $G' \gg G''$ independent of ω and is independent of strain up to a large strain that will break (Figure 3.9d)

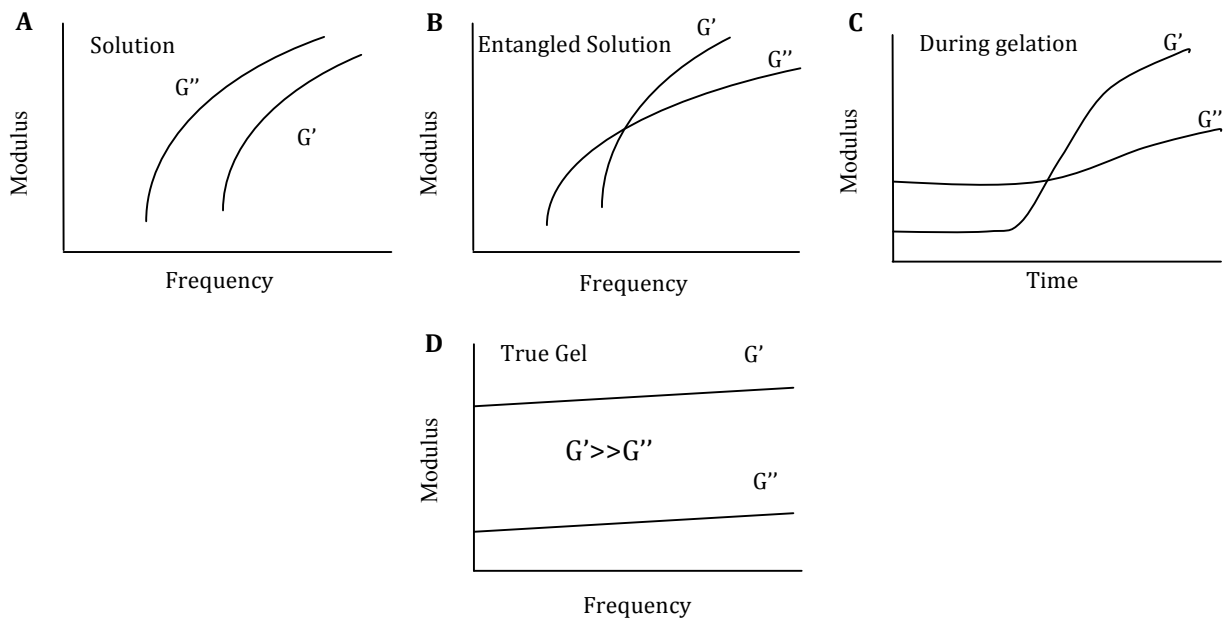


Fig. 3.9 Mechanical spectra of biopolymer systems (a-b) and stages in gelation (c-d) based on the frequency sweep test. Storage modulus (G') and Loss modulus (G''). (Ikeda & Foegeding, 2003)

Controlled Stress Rheometer (Figure 3.10) functions as a sample is placed between the two parts of the fixtures (upper and lower). The rotationally controlled piece transmits an input to the sample and the stationary piece detects the response.

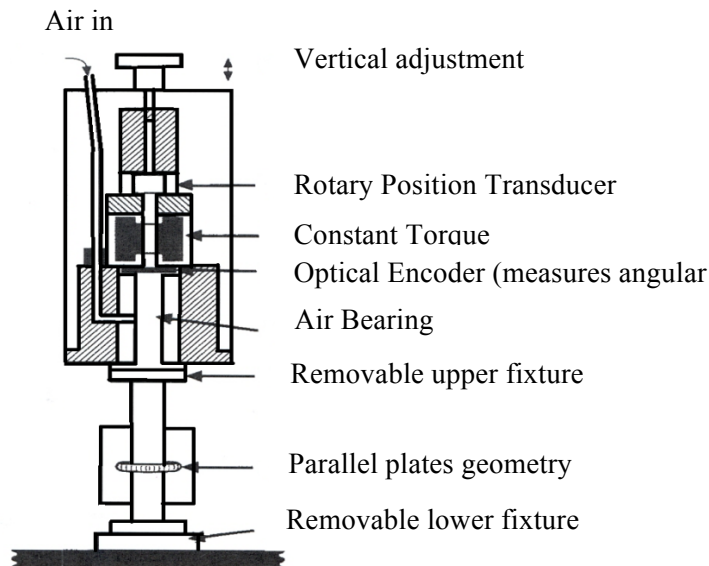


Fig. 3.10 Controlled Stress Rheometer; employs oscillatory shear (Kavanagh & Ross-Murphy 1998)

3.5 BIOPOLYMER COMPRESSION TESTING

Compression test measures the strength, fragility, and toughness of material. Young's modulus calculated from the initial linear portion of the stress-strain curve which represents the toughness of the compressed gel (Gal & Nussinovitch 2007). Although some studies report measurements of strength of hydrogel using compression testing, it is not the best method of measurements for hydrogels, as the hydrogel loses structure under pressure before the failure point.

The strength of the sample, stress at failure (σ_f) can be calculated through force/initial cross-sectional area of the sample (Equation 3.8):

$$\sigma_f = F/A_0 \quad \text{Equation 3.8}$$

The strain at failure (ϵ_f) can be calculated by determining the deformation of specimen at failure/original height of the specimen through Equation 3.9:

$$\epsilon_f = \Delta l / l_0 \quad \text{Equation 3.9}$$

Young's modulus of elasticity can be calculated through Equation 3.10:

$$E = \sigma_f / \epsilon_f \quad \text{Equation 3.10}$$

3.6 BIOCERAMICS USED IN BIOMEDICAL APPLICATIONS

In the past few decades, bioceramics have been widely used in biomedical applications especially in dental and orthopaedic area. They are highly biocompatible and have good chemical stability in the body without producing toxic material even when in contact with body fluid for a long time. They categorize into bioinert ceramics, biodegradable ceramics and bioactive ceramics (Hench & Wilson 1993).

3.6.1 BIOINERT CERAMICS

Typical bioinert ceramics as in Alumina (Al_2O_3) and Zirconia (ZrO_2) implant connects to the natural tissue while a fibrous tissue is formed around the bioinert ceramic after implantation. They have weak bonding strength with the natural tissue and have stable

structure and they do not undergo chemical reaction or biodegradation following implantation (Hayashi et al. 1992; Hayashi et al. 1993).

3.6.2 BIODEGRADABLE CERAMICS

Biodegradable ceramics as in TCP degrade after implantation. So the mechanical strength of the implants diminishes with degradation of the biodegradable ceramics. Therefore, it is important that the implant possesses a high mechanical strength and low degradation rate to be supportive to the defective bone while new bone is being formed (Hench & Wilson 1993).

3.6.3 BIOACTIVE CERAMICS

Bioactive ceramics as in hydroxyapatite (HA), bind to the natural bone through strong chemical bonds. The composition and structural similarity of HA to the inorganic material of natural bone has recently attracted researcher's interest (Pramanik et al. 2005; Nazarpak et al. 2009; Tanner 2010). A recent research by Watanabe et al. (2007) reported the fundamental properties of quick-forming HA/agarose gel composites and their potential as an injectable bone substitute. Promising results were obtained as the composite dissolved rapidly and was replaced by newly formed bone and thus reported HA to be a good candidate as an injectable biomaterial in the fields of orthopaedic, oral and maxillofacial. Further, researches reported HA to reinforce polymer biocomposites for synthetic bone substitutes with tailored mechanical, biological and surgical functions (Roeder et al. 2008).

4. DEGRADATION OF POLYSACCHARIDE HYDROGELS IN CELL CULTURE CONDITIONS

4.1 INTRODUCTION AND AIM

The ability to control mechanical properties of cell encapsulating substrates is very important as substrate stiffness can affect cell growth, viability, migration, and differentiation (Alsberg et al. 2002). Therefore, variation in mechanical properties during cell culture could have an impact on tissue development. Such effects are apparent when culturing MSCs. For example MSCs have recently been shown to differentiate down an osteogenic lineage when encapsulated in stiff materials and down the chondrogenic lineage in a more flexible substrate (Discher et al. 2005; Engler et al. 2006b). As the mechanical properties of the scaffolds used in regenerative medicine is important, a study on the behaviour of various 3D hydrogel networks under *in vitro* culture conditions seems necessary. The current investigation focuses on the effect of the cell culture conditions on the mechanical properties of calcium crosslinked low-acyl gellan gum (0.5% w/v, 1% w/v and 2% w/v), LM pectin (0.5% w/v, 2% w/v and 5%w/v) and alginate (0.5% w/v, 2% w/v and 5%w/v) hydrogels over a period of 28 days incubation. Small deformation oscillatory rheology was used to characterise the gels at different time intervals in acellular samples and in Rat Bone Marrow Stromal Cells (rBMSC) seeded samples. In addition, differentiation of rBMSCs towards the osteogenic lineage was investigated within the selected concentrations of hydrogels (alginate 2%w/v, pectin 2%w/v and gellan gel 1%w/v). We hypothesized that supplemented growth media increases the degradation rate of the gels. We also theorized that due to the physiochemical structure of the gels, sodium alginate gel and pectin would degrade faster than gellan gel.

4.2 MATERIALS AND METHODS

Low-acyl Gellan Gum (Gelrite) was from Sigma-Aldrich Company Ltd., Dorset, UK, with an average MW of 1,000,000. LM pectin (GENU® LM type: X-914-02) with a very low (<32%) DE was from FMC biopolymer, and the low viscosity (20- 40 cps), sodium alginate with an M:G ratio of 1.56 was from Sigma-Aldrich Company Ltd., Dorset, UK. Calcium chloride dihydrate was from Fisher Scientific. Unless specified, all other materials were obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

4.2.1 PREPARATION OF POLYSACCHARIDE SOLUTIONS

All polysaccharide solutions were prepared by dissolving in deionized water (dH₂O) at 85°C with continuous stirring to a concentration of (0.5, 1, 2% w/v) gellan gum, (0.5%, 2%, 5% w/v) alginate and (0.5, 2, 5% w/v) LM pectin. The prepared polysaccharide solutions were all autoclaved in identical conditions before they were used to make hydrogels.

4.2.2 ACELLULAR HYDROGEL PREPARATION

The polysaccharide solutions were poured into disc-shaped moulds and covered with filter paper impregnated with 100mM CaCl₂. The samples were then incubated at 37°C for 3 hours to form crosslinked hydrogel discs which were 20 mm in diameter and 3mm in height. The disc-shaped hydrogels were then washed three times in non-supplemented Dulbecco's modified Eagle's medium (SDMEM) or dH₂O to remove excess CaCl₂ before incubating in supplemented media (SDMEM) or dH₂O.

4.2.3 ISOLATION AND CULTURE OF rBMSCs

rBMSCs were isolated from female Wistar rats following sacrifice. The femurs and tibiae were removed and the remaining soft tissues were detached aseptically. Both ends of the femur and tibia were removed and the marrow cavity was flushed out with sterile phosphate buffered saline (PBS) using a 20-gauge needle. The resulting cell suspension was centrifuged at 1000g for 5 minutes. The cell pellet was washed in phosphate buffered saline (PBS) and centrifuged at 1000g for a further 5 minutes. The PBS was removed and the resulting cell pellet was re-suspended in SDMEM (Invitrogen, Paisley, UK) supplemented with 10% (v/w) Foetal Bovine Serum (FBS) (Biosera, Ringmer, UK) and 1% (v/w) penicillin and streptomycin (Invitrogen, Paisley, UK). Cells were cultured in 15cm diameter tissue culture plates at 37°C (95% air, 5% O₂) until 70-80% confluent and then split at a ratio of 1:4.

4.2.4 ENCAPSULATION AND CULTURE OF rBMSCs WITHIN HYDROGELS

The cells expanded to passage 5, were dispersed in the polysaccharide solutions at a density of 0.76×10^6 cells mL⁻¹ and then poured into disc-shaped moulds and treated the same as described above for the acellular samples. The cell-loaded hydrogels were then cultured in osteogenic media to facilitate the rBMSCs differentiation to osteoblasts. The osteogenic media composed of Alpha Minimum Essential Medium Eagle (α -MEM) supplemented with 10% FBS (PAA, Somerset, UK), 1% Penicillin, 2.2% HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2% L-Glutamine, 10^{-8} M dexamethasone, 10^{-2} M β -glycerophosphate, and 10^{-4} M ι -Ascorbic acid 2-phosphate sesquimagnesium salt hydrate magnesium. The cell culture media was replaced with fresh media every three days during the culture period.

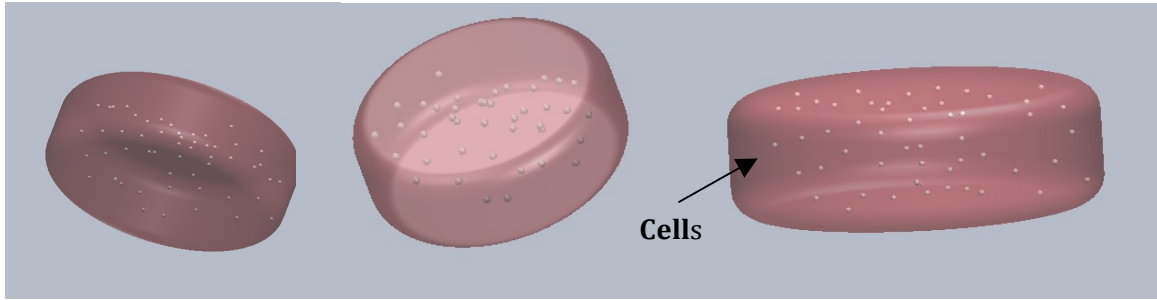


Fig. 4.1 Encapsulated cells in hydrogels incubated in SDMEM

4.2.5 RHEOLOGICAL MEASUREMENTS

The mechanical properties exhibited by the hydrogels were determined using oscillatory rheological measurements of storage modulus (G'), loss modulus (G''), and dynamic viscosity (η^*) at 37°C using a stress-controlled rheometer (TA instruments). The gels were characterized using 20 mm stainless steel parallel plate geometry (sand blasted to prevent slippage) at 0.5% strain, across a frequency (ω) range of 1 rad/s to 100 rad/s at a constant temperature of 37°C. Prior to loading, the gelled disk was trimmed to size using a razor blade and the thickness of the gels corresponded to the geometry gap (3mm). All tests were performed in triplicate, and mean values and corresponding standard deviations calculated.

4.2.6 HISTOLOGY

Samples were removed from their culture media and submerged in 4% formaldehyde solution (Sigma-Aldrich Company Ltd., Dorset, UK) for two days at 4°C. Samples were then stored in sterile PBS at 4°C before being embedded in Cryo-M-BED (Bright, UK). Samples were sectioned to 10 μ m thickness using a Cryostat (Bright, Starlet 2212, UK). Sections were then stained using Alizarin Red Staining for 3 minutes. Samples were

then dehydrated first in acetone followed by acetone-xylene (1:1) solution. Stained sections were then visualised using a light microscope (Nikon). Negative and positive control samples were prepared with the incorporation of nanocrystalline HA powder into the hydrogels set using 100mM CaCl₂. Monolayer samples and the control samples were prepared following the same protocol with the exception of sectioning in the monolayer rBMSCs where the cells were grown in 6-well plates which were washed with sterilised PBS three times before being stained.

4.2.7 QUANTITATIVE ANALYSIS OF MINERAL FORMATION

Quantification of mineral formation was undertaken using image analysis of the histological sections in each hydrogel. Five randomly selected images of various field widths were threshold using image J software (NIH, Maryland, USA) and converted to a binary image. The percentage of the mineral per unit area was calculated in each gel and the weight of mineral then calculated using the density of 3.16 gcm⁻³ for HA (Elliott et al. 1994). The quantity of mineral produced per seeded cell was calculated from the seeding density of 0.76x10⁶ cells per ml presuming mitotic inhibition (Hunt et al. 2009).

4.2.8 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used for statistical analysis, followed by a Tukey post-hoc test to determine the statistical significance ($p < 0.05$) between test groups (SPSS v.17, Chicago, USA).

4.3 RESULTS

4.3.1 VISUAL INSPECTION

To assess any visual and textural differences in the hydrogels over the period of incubation, the samples were analyzed texturally. Following the 28 days of incubation in osteogenic media, SDMEM and dH₂O, all hydrogel samples retained self-supporting, disc-shaped morphology (Figures 4.2-4.4). The pink staining of the media incubated samples was due to the presence of phenol red pH indicator in the SDMEM. Hydrogel samples incubated in osteogenic media at chosen concentrations retained self-supporting, disc-shaped morphology just like the other samples incubated in SDMEM and dH₂O.

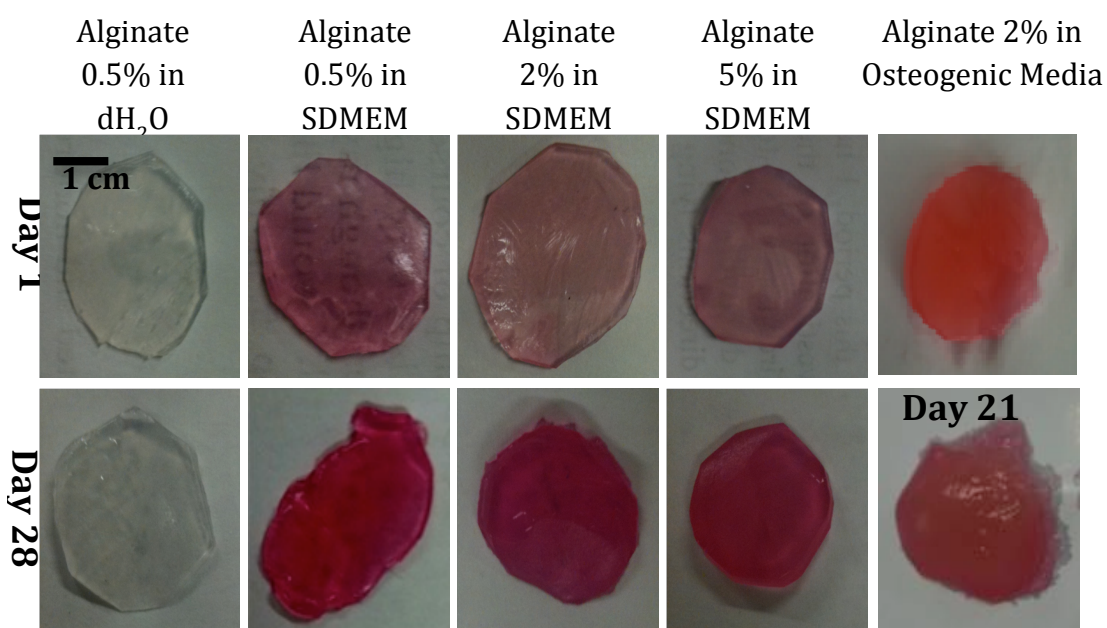


Fig. 4.2 Images of Alginate hydrogel samples incubated in dH₂O, growth media (SDMEM) at day 1 and 28, also osteogenic media at day 21.

From inspection of the images of the pectin samples, the pH of the gel seemed to be slightly acidic as the colour of the gels changed from red to orange. Moreover, textural observations of LM pectin hydrogel samples appeared fragile (Figure 4.3).

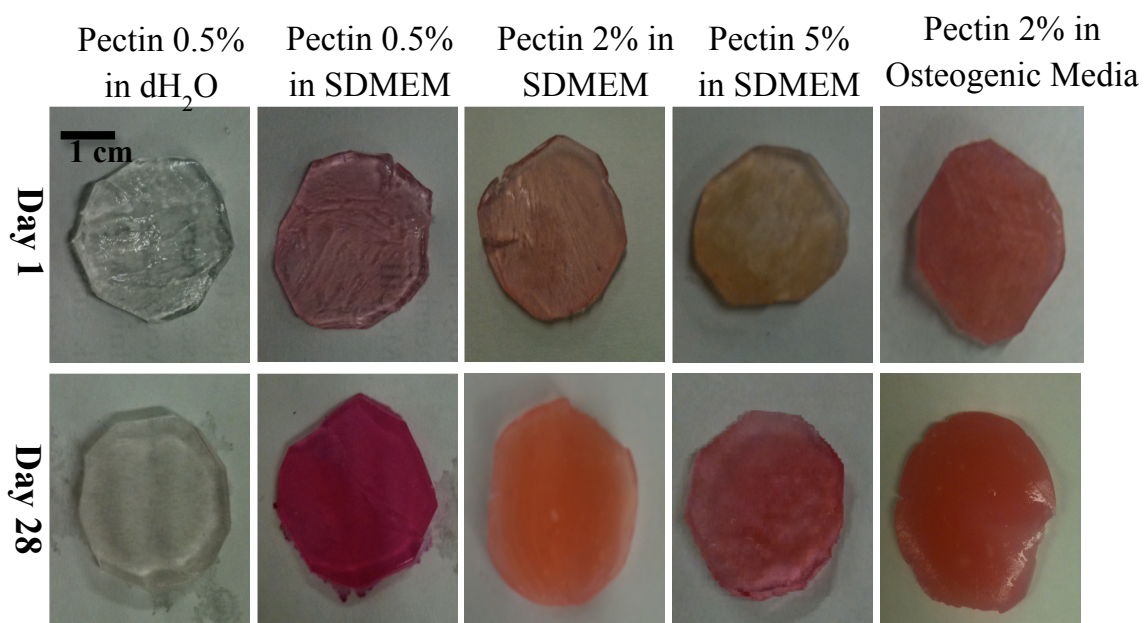


Fig. 4.3 Images of Pectin hydrogel samples incubated in dH₂O, growth media (SDMEM), and osteogenic media at day 1 and 28.

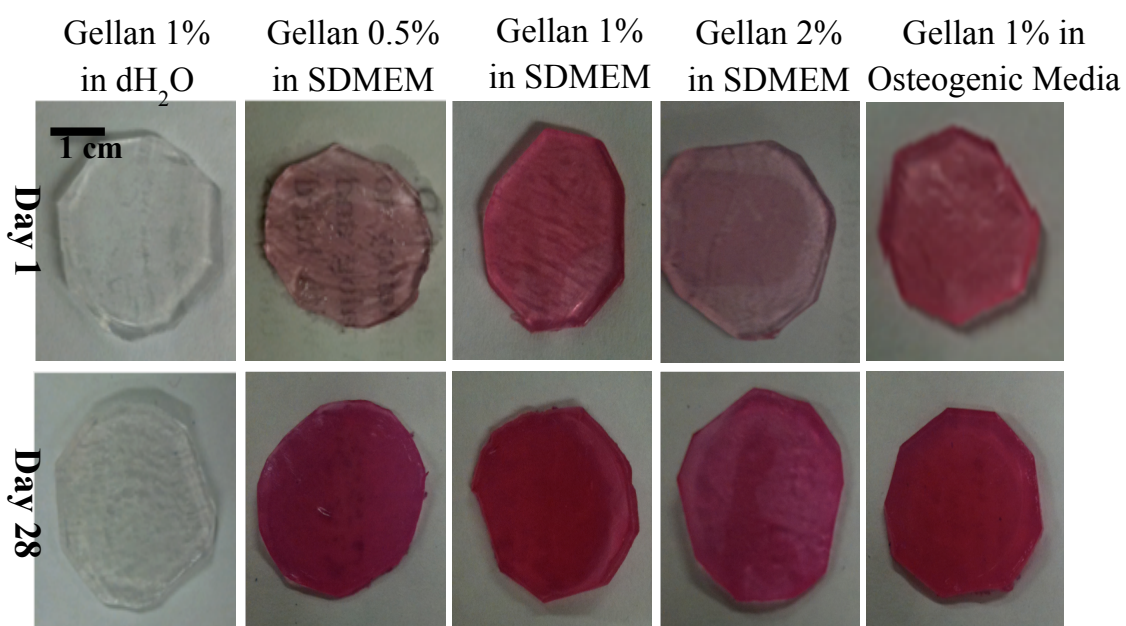


Fig. 4.4 Images of Gellan hydrogel samples incubated in growth media (SDMEM), dH₂O and osteogenic media at day 1 and 28

4.3.2 RHEOLOGICAL ANALYSIS OF DEGRADATION

The low-acyl gellan gel samples appeared less fragile in comparison to the LM pectin

gel whereas the alginate gel was very difficult to handle after 21 days of incubation in both the cellular samples and the acellular samples in SDMEM (Figure 4.4).

Rheological measurements of G' , G'' and η^* were taken to evaluate the properties of 0.5, 2 and 5% w/v alginate; 0.5, 2 and 5% w/v LM pectin and 0.5, 1 and 2% w/v gellan gum gels and to assess the effect of encapsulation of rBMCs on these hydrogels. The dynamic shear moduli G' and G'' of each hydrogel was measured and the reduction of G' (measured at an angular frequency of 10 rad s^{-1} 0.5% strain 37°C) over the 28 day is represented in Figure 4.5-4.9 as a percentage of original modulus.

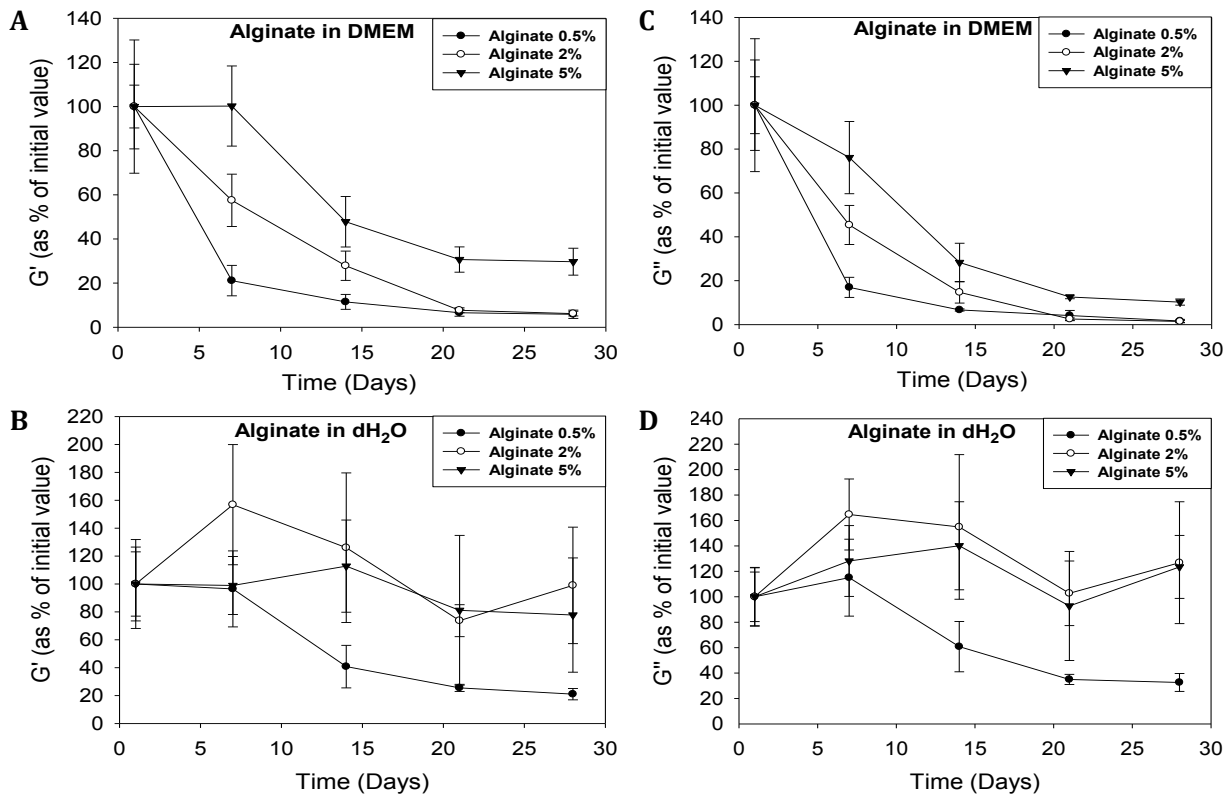


Fig. 4.5 Percentage changes in G' (a,b) and G'' (c,d) (10 rad s^{-1} ; 0.5% strain; 37°C) of acellular alginate samples of 0.5% w/v (filled circles), 2%w/v (open circles) and 5% w/v (filled triangles) over 28 days study incubated in SDMEM (a,c), and dH₂O (b,d). Vertical error bars represent the standard deviation of the reported mean values (n=3).

Figure 4.5 shows measurements of (a,b) G' and (c,d) G'' at 0.5% strain 10rad/sec for different concentration alginate hydrogel samples incubated in both SDMEM and dH₂O for a period of 28 days.

The 0.5% w/v concentration alginate samples (Figure 4.5) incubated in both SDMEM and dH₂O represented a dramatic degradation rate falling to about 10% of its original value in SDMEM and 40% in dH₂O at day 14. As the concentration of the gel increased, the rate of degradation slowed down as is apparent in Figure 4.5a and b to about 30% of the original value for alginate 2% w/v and to about 45% of the original value for alginate 5% w/v both in SDMEM on day 14. Alginate samples of 0.5% and 2% w/v almost degraded fully by day 28 (95% degradation), this is while alginate 5% w/v samples measured on the same day represented a higher modulus as they retained 40% of their original strength. Degradation was not marked in the higher concentration samples incubated in dH₂O ($p < 0.05$) throughout the 28 day study.

Similar trend is apparent in the pectin samples. Figure 4.6 (a-d) incubated in SDMEM and dH₂O over 28 days. However, the critical difference is that pectin sample across all the three concentrations (0.5%, 2% and 5% w/v) have shown a much slower degradation rate with the maximum degradation of 60% on day 28.

This is while the degradation rate in alginate was 95% measured on day 28. The 0.5% w/v samples at day 14 retained about 70% of their original value and to the same extent as pectin gel concentration increased the gel degradation rate decreased. The remarkable increase in the modulus of the 5% w/v pectin samples from the day 21 to 28 is presumed to be due to both experimental error and media changes one day prior

to measurements which would result in the flux of a large amount of cations present in the media into the gel matrix.

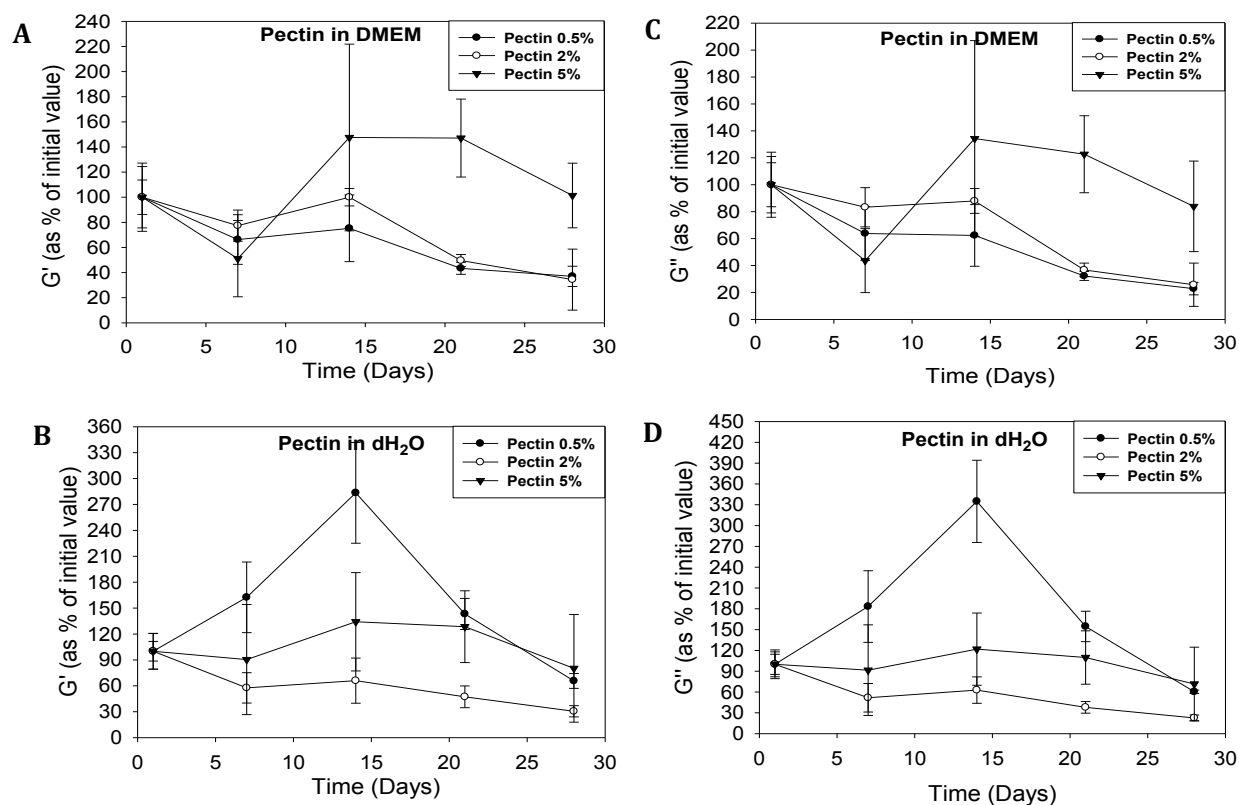


Fig. 4.6 Percentage changes in G' (a,b) and G'' (c,d) (10 rad s^{-1} ; 0.5% strain; 37°C) of acellular pectin samples of 0.5% w/v (filled circles), 2%w/v (open circles) and 5% w/v (filled triangles) over 28 days study incubated in SDMEM (a,c), and dH₂O (b,d). Vertical error bars represent the standard deviation of the reported mean values ($n=3$).

Pectin samples incubated in dH₂O surprisingly showed a high degradation rate which is shown in Figure 4.6b, since this was unexpected, the measurements were repeated. When repeated, there was no significant gel degradation ($p < 0.05$) (Figure 4.7b), which highlights the sensitivity of these materials to small changes in culture conditions.

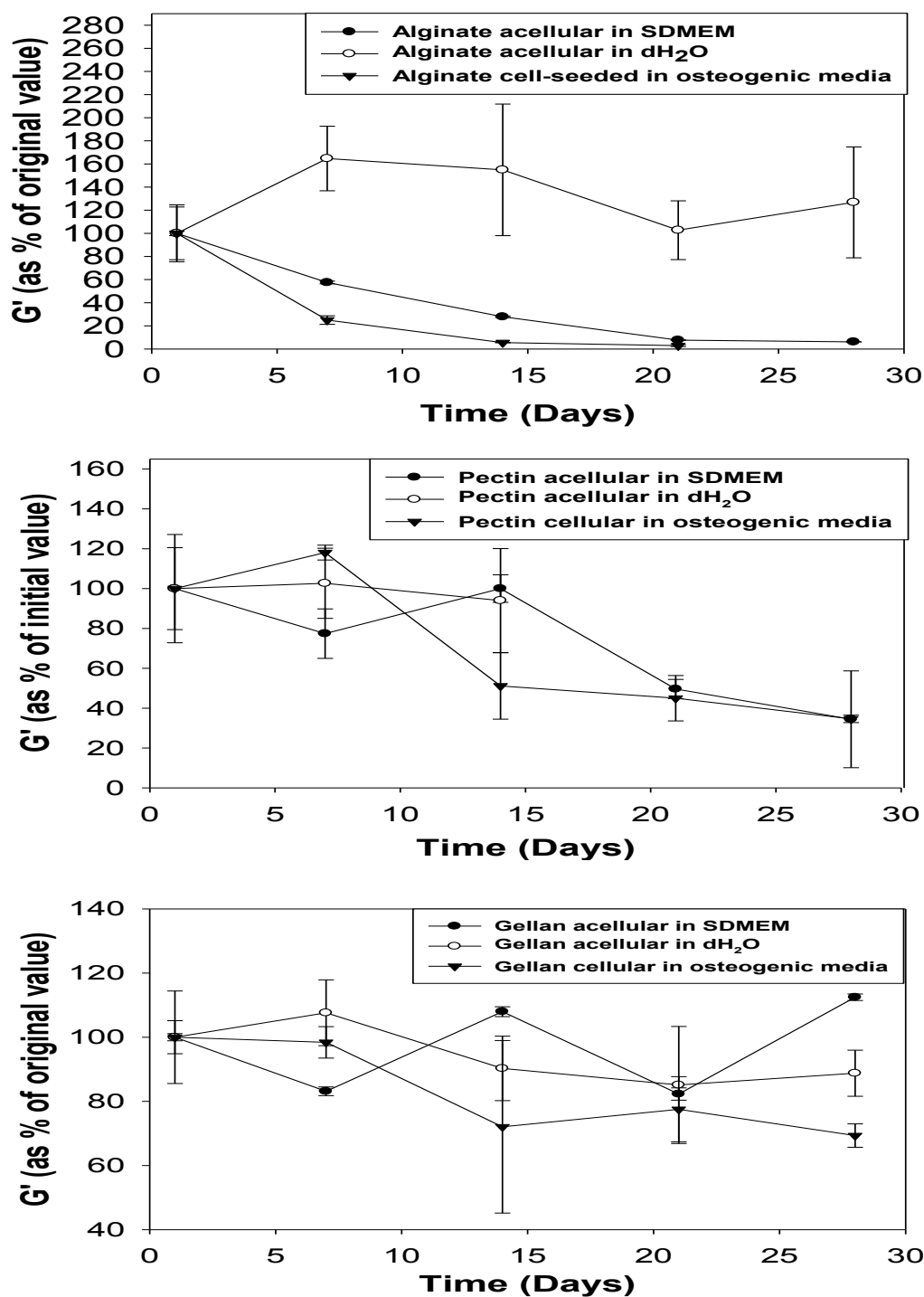


Fig. 4.7 Percentage reduction in G' (10 rad s^{-1} ; 0.5% strain; 37°C) of Alginate $2\%w/v$ (a), Pectin $2\%w/v$ (b) and Gellan gel $1\% w/v$ (c) over a 28 day study incubated in osteogenic media (filled triangles) rBMSCs seeded, acellular samples incubated in SDMEM (filled circles), and acellular samples incubated in dH_2O (open circles). Vertical error bars represent the standard deviation of the reported mean values ($n=3$).

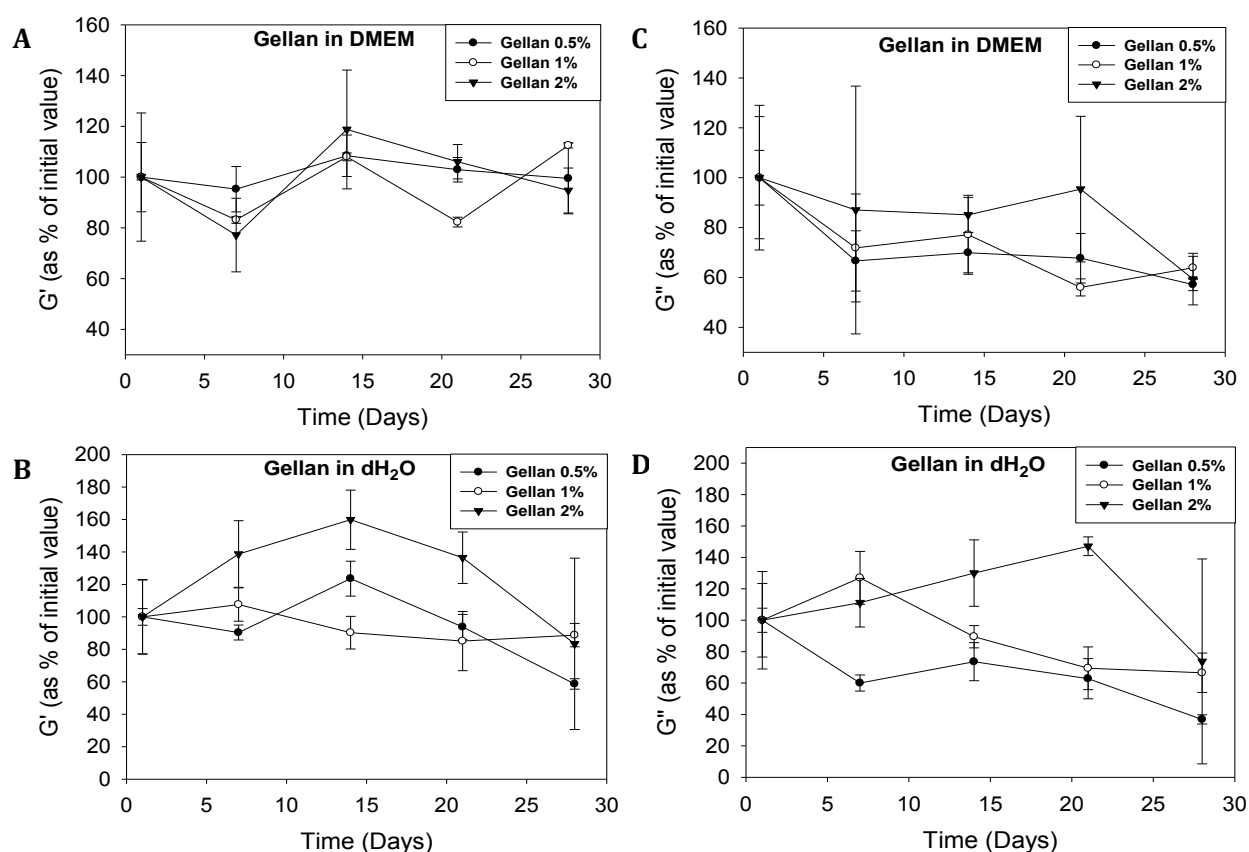


Fig. 4.8 Percentage changes in G' (a,b) and G'' (c,d) (10 rad s^{-1} ; 0.5% strain; 37°C) of acellular gellan samples of 0.5% w/v (filled circles), 1%w/v (open circles) and 2% w/v (filled triangles) over 28 days study incubated in SDMEM (a,c), and dH₂O (b,d). Vertical error bars represent the standard deviation of the reported mean values (n=3).

A slightly different trend was observed in the gellan samples incubated in SDMEM and dH₂O in comparison to the alginate and pectin samples. The graphs in Figure 4.8 illustrate this significant difference. The degradation rate slightly fluctuates across the three concentrations in G' and appear more stable in G'' . Gellan maintained its high modulus throughout the 28 day study with minimum rate of degradation. A similar trend was visible in the gellan samples incubated in dH₂O.

Figures 4.7 and 4.9 compare the degradation rate of 2% w/v alginate, 2% w/v pectin and 1% w/v gellan hydrogel samples rBMSC-seeded (cellular) and without cells (acellular) incubated in SDMEM, dH₂O and osteogenic media. The rBMSC-seeded alginate hydrogels demonstrated a dramatic degradation rate when incubated in osteogenic media (Figure 4.7a) with G' reduced to 20% of the original value at day 7 and by day 28, the samples were too fragile to measure. Degradation was also witnessed in the acellular samples cultured in SDMEM however this was at a slower rate and the gel retained 60% of its original G' at day 7. At day 21, there was no significant difference ($p < 0.05$) between the cellular and acellular samples incubated in media (Figure 4.7a). Samples incubated in dH₂O showed no significant changes in G' over the period of the study. rBMSC-seeded pectin gels also showed a reduction in G' during the 28 day study. At 14 days when incubated in osteogenic media, the reduction in G' was ~55% with no degradation apparent in the acellular SDMEM pectin. By day 21 however, there was no difference between the cellular samples and the acellular samples incubated in SDMEM with the G' of both reduced by ~50% at day 21 and 60% at day 28 (Figure 4.7b). The gellan gum samples showed no significant degradation during the study in the acellular samples incubated in media and SDMEM. Interestingly, G' of the cellular hydrogel sample in osteogenic media reduced strength to 40% of its original value at day 14. It then recovered to ~85% of its strength by day 21 and at 28 days retaining 80% of its original value (Figure 4.7c). When comparing the degradation of the three hydrogels in dH₂O, Figure 4.9a illustrates that even after a period of 28 days, no significant change ($p < 0.05$) in G' was observed. The acellular samples incubated in SDMEM (Figure 4.9b) show very clear differences between the hydrogels. Both alginate and pectin appear to exhibit a similar degradation pattern

however pectin degraded at a much slower rate whereas gellan seems to fluctuate by $\pm 15\%$ over the incubation period. A similar trend is witnessed in the cellular samples (Figure 4.9c) with degradation occurring most rapidly in the order alginate > pectin > gellan.

The mechanical spectra of the three hydrogels at day 1 and day 28 (day 21 in the alginate) in the three incubation conditions are given in Figures 4.10-4.12. It is clear that at day 1 all hydrogels show true gel mechanical spectra with $G' \gg G''$ and a linear reduction in η^* . When day 1 mechanical spectra (Figure 4.10a) are compared to spectra at day 21 (Figure 4.10b), in the cellular alginate samples values of G' and G'' are reduced by an order of magnitude and at higher frequencies G'' becomes greater than G' . The acellular samples (Figures 4.10c and 4.10d) also show a drop in both moduli but not to the same extent as occurred in the cellular sample. It is also evident that at day 28, G' begins to decrease and G'' begins to increase at higher frequencies (Figure 4.10d). Reductions in moduli from those at day 1 (Figure 4.11a) are also seen in the cellular pectin samples following 28 days incubation (Figure 4.11b) and to a lesser extent the acellular samples in SDMEM (Figures 4.11c and 4.11d). However these decreases are not of the order of magnitude seen in the alginate gels and no frequency dependent changes in moduli were observed. The mechanical spectra of the gellan samples show little variation between day 1 and day 28 in the cellular samples (Figures 4.12a and 4.12b) and the acellular samples (Figures 4.12c and 4.12d).

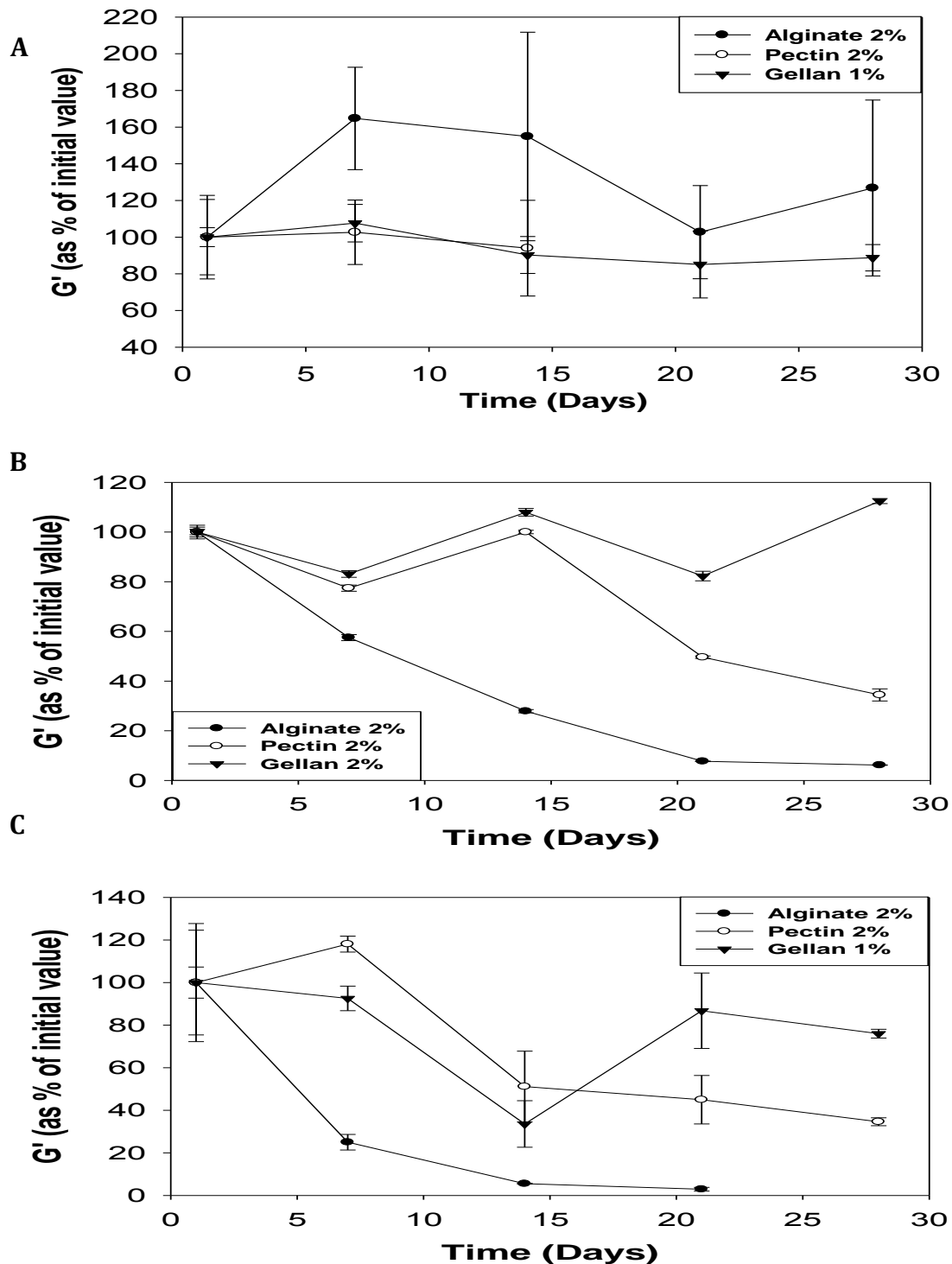


Fig. 4.8 Percentage reduction in G' of (a) acellular Alginate, Gellan and Pectin cultured in dH₂O, (b) acellular hydrogels cultured in SDMEM, and (c) cell-seeded hydrogels cultured in Osteogenic media (10 rad s⁻¹; 0.5% strain; 37°C), over a 28 day incubation for 2% w/v Alginate (filled circles), 2% w/v Pectin (open circles), and 1% w/v Gellan (filled triangles). Vertical error bars represent the standard deviation of the reported mean values (n=3).

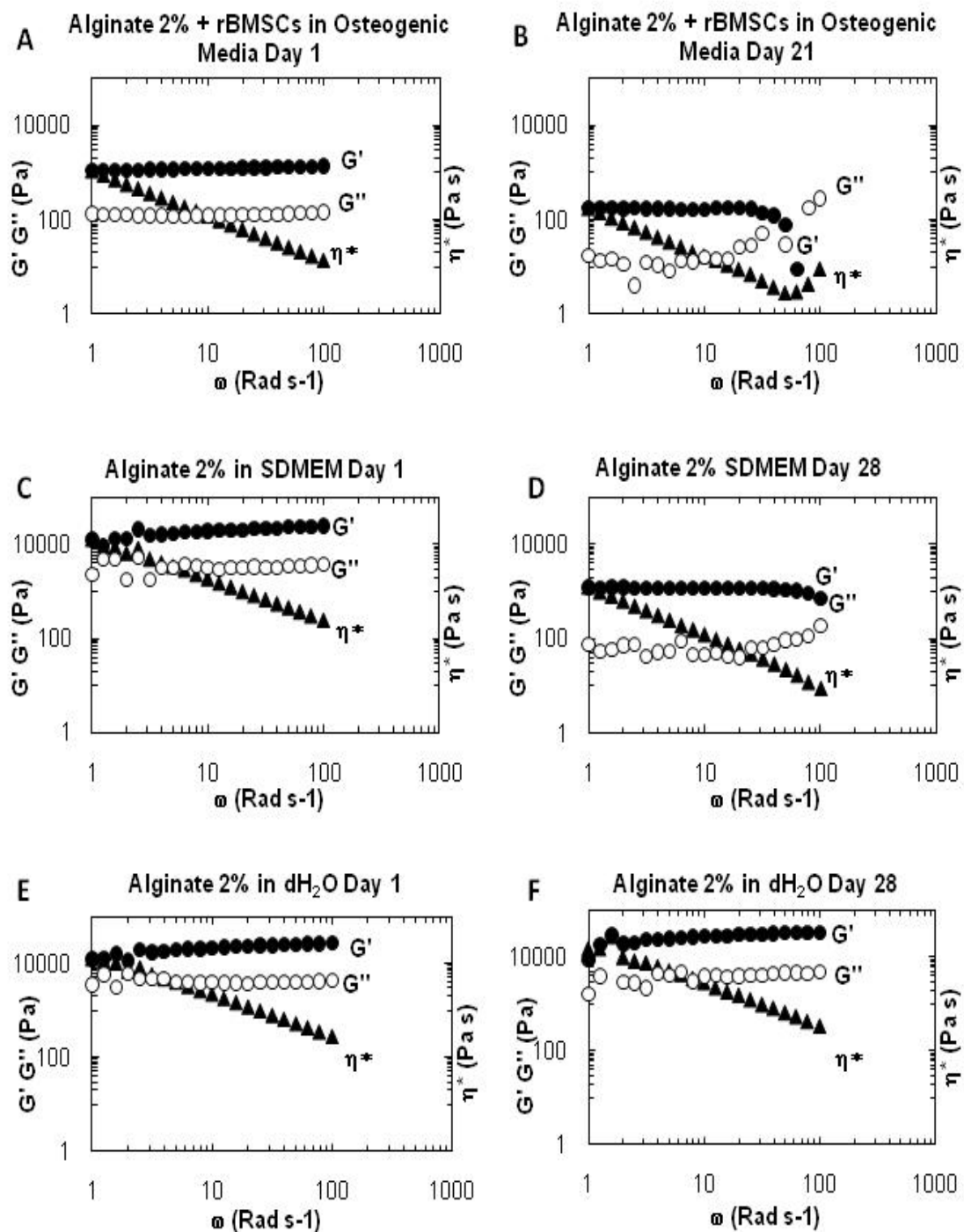


Fig. 4.9 Mechanical spectra (0.5% strain; 37°C), at day 1 and 28 showing variation of G' (filled circles), G'' (open circles) and η^* (filled triangles) with angular frequency for 2% w/v alginate hydrogel (a,b) containing encapsulated rBMSCs incubated in osteogenic media, (c,d) acellular samples incubated in SDMEM, and (e,f) acellular samples incubated in dH₂O. Alginate in osteogenic media lost its mechanical properties from day 21 on and could not be measured.

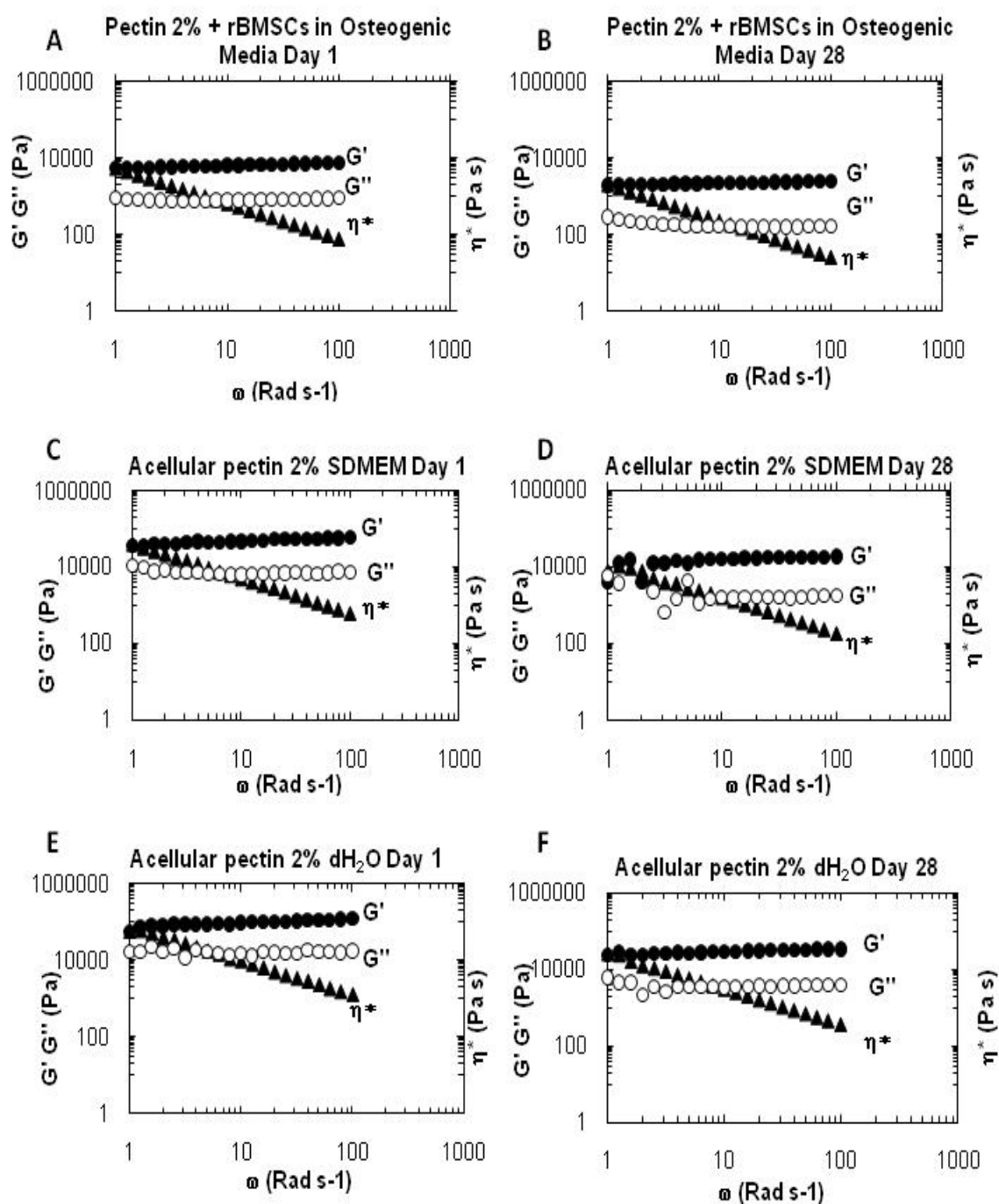


Fig. 4.10 Mechanical spectra (0.5% strain; 37°C), at day 1 and 28 showing variation of G' (filled circles), G'' (open circles) and η^* (filled triangles) with angular frequency for 2% w/v pectin hydrogel(a,b) containing encapsulated rBMSCs incubated in osteogenic media, (c,d) acellular samples incubated in SDMEM, and (e,f) acellular samples incubated in dH₂O.

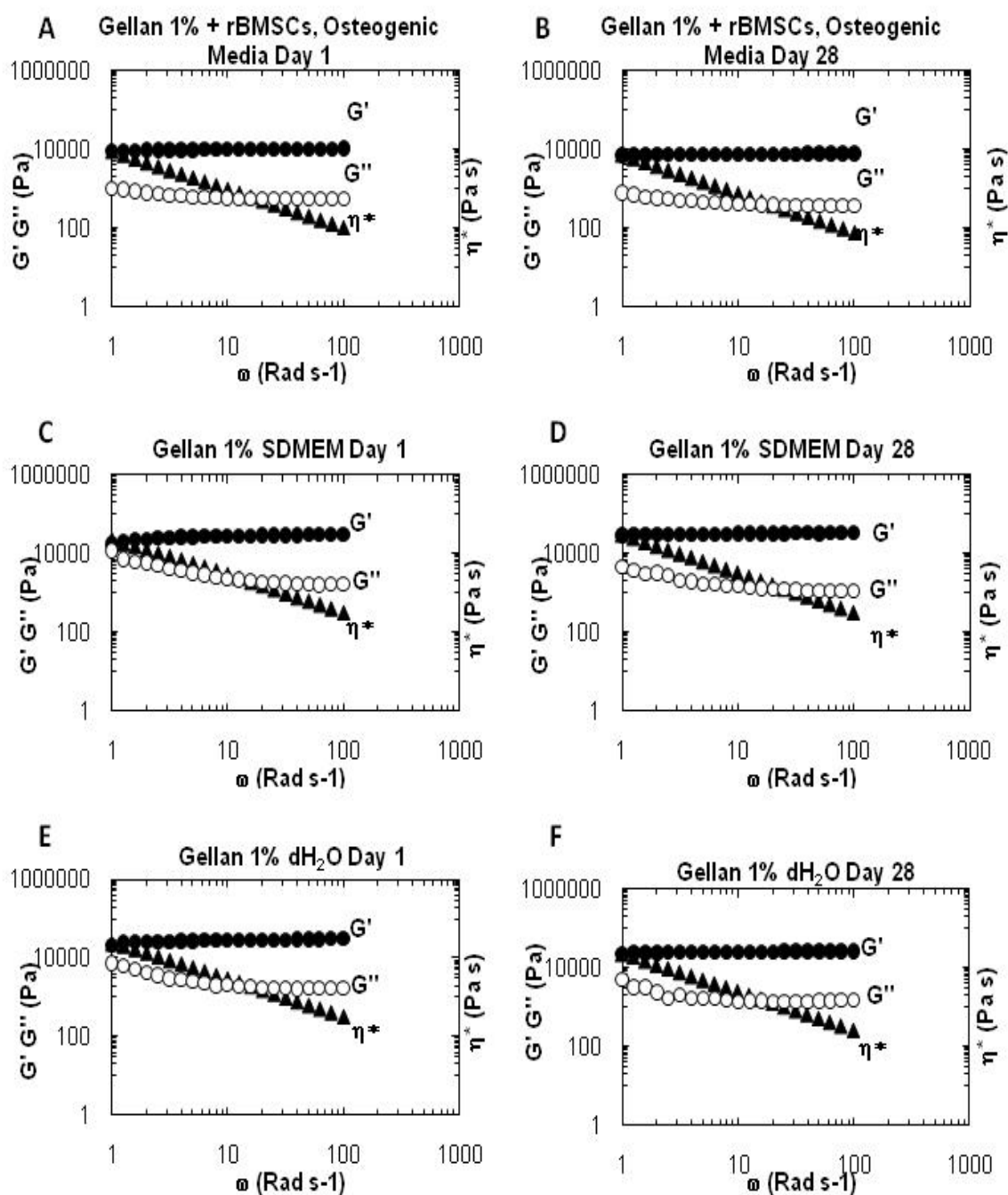


Fig. 4.11 Mechanical spectra (0.5% strain; 37°C), at day 1 and 28 showing variation of G' (filled circles), G'' (open circles) and η^* (filled triangles) with angular frequency for 1% w/v gellan hydrogel (a,b) containing encapsulated rBMSCs incubated in osteogenic media, (c,d) acellular samples incubated in SDMEM, and (e,f) acellular samples incubated in dH₂O.

4.3.3 DIFFERENTIATION AND MINERALISATION OF rBMSCs

To verify the ability for the multipotent rBMSCs used in this study to differentiate into osteoblasts, the cells were cultured in osteogenic media in monolayer for 21 days. When the cells were ~75% confluent a sample was stained with Alizarin Red. The micrograph in Figure 4.13 provides evidence of mineral deposition highlighted by the dark red nodules indicating that the rBMSCs had differentiated into osteoblasts which had begun to produce bone mineral.

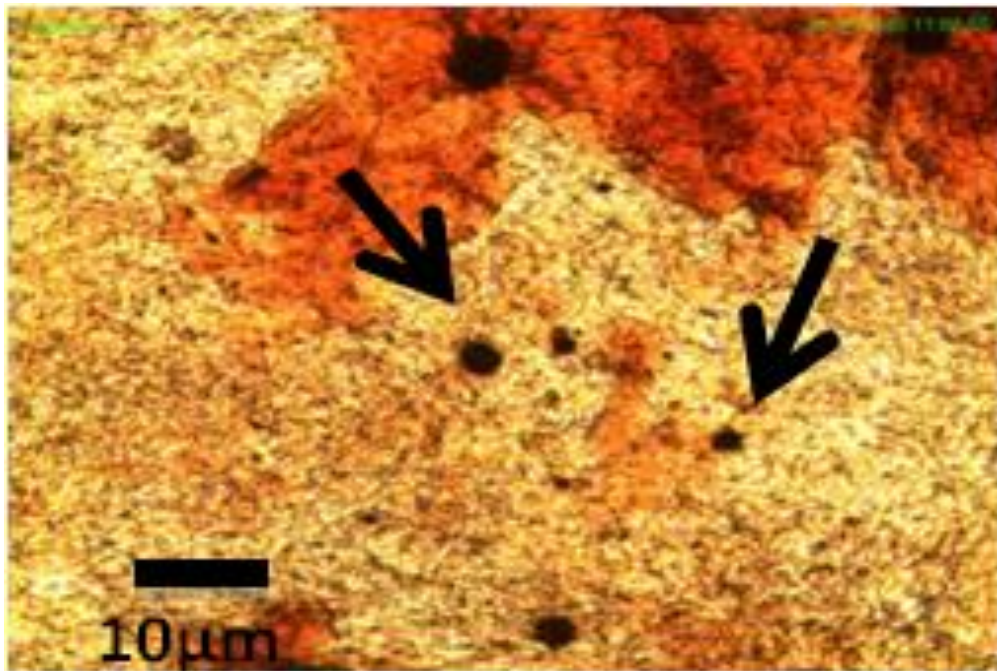


Fig. 4.12 rBMSCs seeded at 75% confluence and cultured in the presence of osteogenic supplements on polystyrene for 21 days. Mineral deposits were detected using the Alizarin red staining; the mineral appears as focal dark red spots.

A similar procedure was performed in the hydrogel samples where 10µm sections of the gels were stained with Alizarin red following a 28 day culture in osteogenic media. It was apparent that nodules of mineral were deposited through the three different

hydrogels (Figures 4.14a-c). These mineral deposits were not present in the acellular control samples an example of which is shown in Figure 4.14d. As a positive control, HA which is the main component of bone mineral was loaded into the hydrogels before 10µm sections were stained with Alizarin red. The HA loaded alginate and gellan is shown in Figure 4.14e and 4.14f respectively, where the HA is clearly visible as dense dark red areas in the gels.

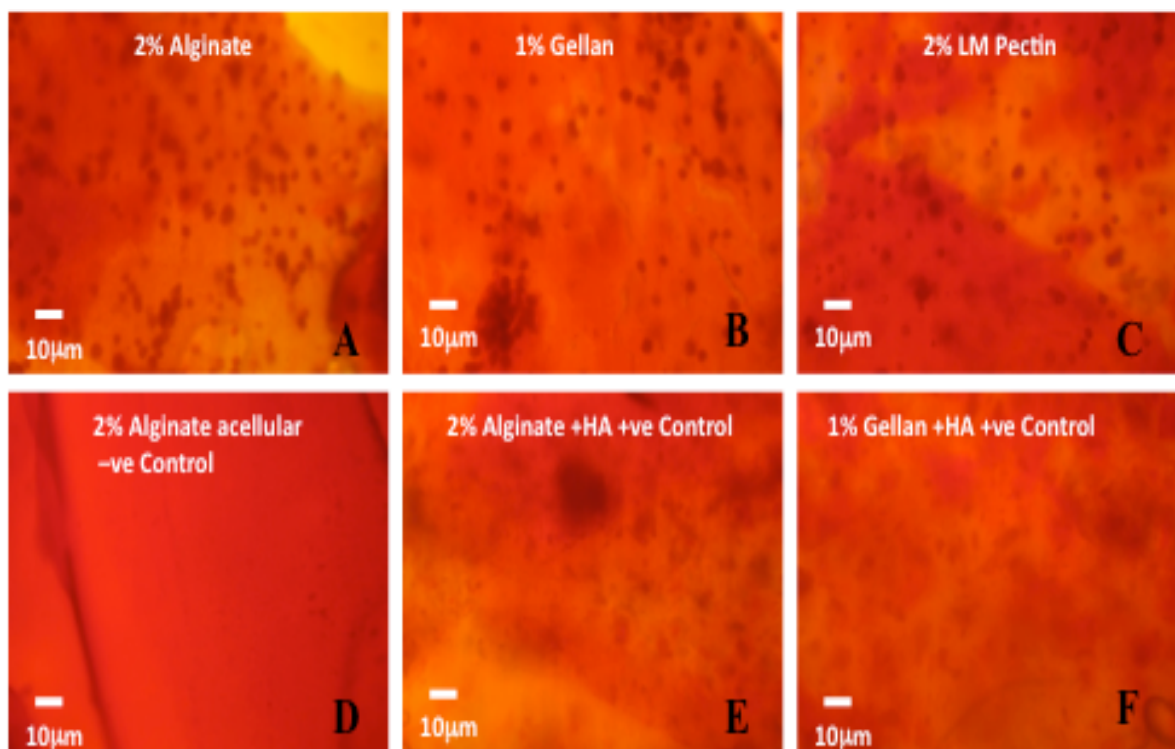


Fig. 4.13 (a,b,c) Images of the gels after culture for 21 days stained with Alizarin Red. Calcium deposits within the hydrogel structures (red nodules) indicated mineralisation in all the gel samples. Reference samples (d) negative control acellular sample and positive control samples of (e) alginate and (f) gellan loaded with HA.

The mineral content in each hydrogel was quantified using image analysis and the results are shown in Figure 4.15. Although there was greater measured mineral

content in the alginate and pectin samples compared with that of the gellan sample, this difference was not significant ($p < 0.05$). From this data the mass of mineral produced per cell was calculated to be $1.13\mu\text{g}/\text{cell}$ for alginate, $1.11\mu\text{g}/\text{cell}$ for pectin and $0.9\mu\text{g}/\text{cell}$ for gellan.

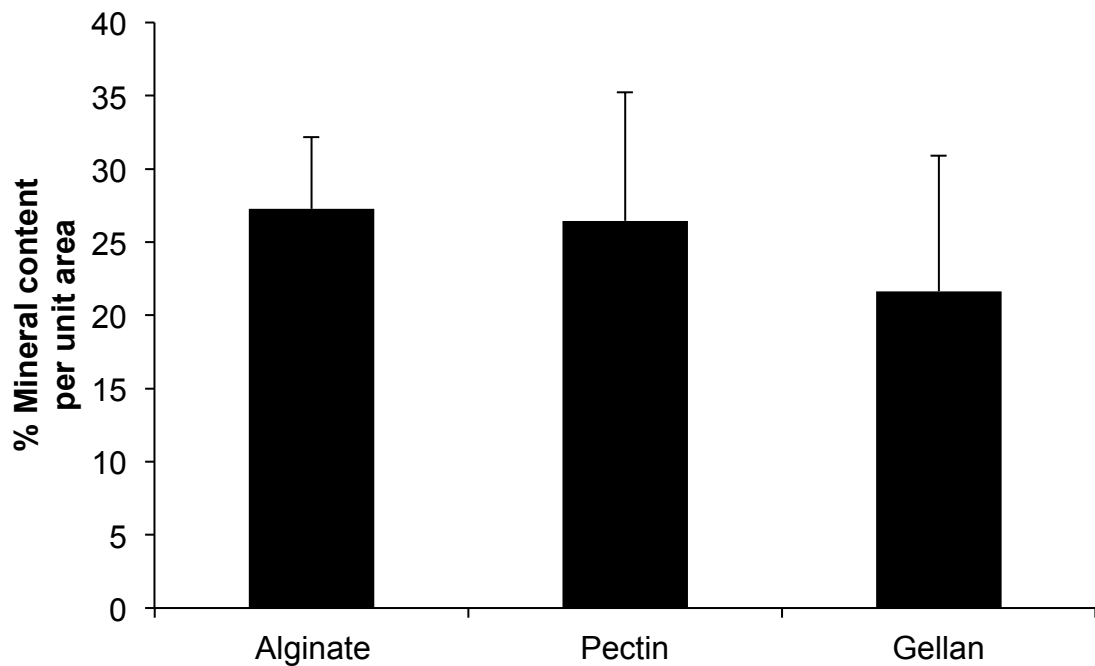


Fig. 4.14 Percentage of mineral per unit area at 21 days for alginate, pectin and gellan measured using image analysis on histological sections. Results represent mean values, with vertical error bars representing the standard deviation between 5 randomly chosen areas of the sample.

4.4 DISCUSSION

The effect of increase in the modulus of alginate gel with an increase in the concentration of the gel is reported by LeRoux (1999) which is attributed to the greater availability of G-blocks which leads to higher crosslink density between G-

blocks and calcium ions (Leroux et al. 1999). This supports the results obtained for the increase in the alginate gel degradation rate as the concentration decreased from 5% w/v to 0.5% w/v.

It is important that the hydrogels used for the purpose of 3D cell culture to retain their mechanical integrity in the culture conditions. Alginate hydrogels, which have been widely used for 3D mammalian cell culture, have previously shown to degrade over time in the presence of cell culture media (Hunt et al. 2010) which may or may not be advantageous depending on the application. In this study we have highlighted that similar less utilised polysaccharide hydrogels such as LM Pectin and gellan gum can sustain cell populations in a manner analogous to alginate whilst having vastly different degradation profiles and organoleptic properties.

The reduction in mechanical properties of the alginate hydrogels when in culture media or in the presence of cells (Figure 4.8 a) is a previously reported phenomena which can be explained by ion exchange between calcium ions which crosslink the alginate and the sodium and potassium present in the media (Donati et al. 2009; Hunt et al. 2010; Rowley et al. 1999) in addition to organic phosphates which are also known as a strong chelators of calcium (Luttrell 1993). The latter may explain the increased rate of degradation witnessed in the cellular samples (Figure 4.8a) especially in osteoblasts producing bone mineral. It would be easy to presume that similar behaviour would be expected in the LM pectin samples as the Ca^{2+} mediated gelation is similar to that of alginate with mechanism described by the “egg box” model (Grant et al. 1973; Morris et al. 1978). The results obtained in this study however, show differences in organoleptic properties (Figures 4.2-4.4) and degradation behaviour over the 28 day culture period (Figure 4.8b). The orange coloured appearance of the

pectin gels indicate slight acidity within the gel which is perhaps caused by the presence of acetic acid, phenol acids substituents on a small proportion of the galacturonate residues which are not present in alginate. When alginate gels are incubated in cell culture conditions, the critical ion exchange step and subsequent reordering of the gel network is thought to occur within the first 7 days following which the gel becomes stabilised (Drury et al. 2004). This hypothesis is further supported with the reduction in G' presented in (Figure 4.8a) and the mechanical spectra at day 21 showing evidence of failure at high frequencies (Figure 4.10b). This does not however explain the slower degradation rate in LM pectin (Figure 4.8b). It is likely that the degradation witnessed in both acellular and cellular LM pectin samples is due to dissipation of calcium ions similar to alginate, however, the more complex structure of pectin may stabilise the structure. The binding residues in alginate (guluronate) generally occur in a block wise manner whereas LM pectin is usually produced by de-esterifying HM pectin. The chemical de-esterification process causes a more random distribution of Ca^{2+} binding galacturonate residues (Winning et al. 2007). The random distribution pattern of LM pectin is likely to introduce shorter, more numerous egg box structures to destabilise. There is also MW and the DE of the pectin to consider. The high MW, low viscosity alginate used in this study is likely to have fewer calcium binding sites compared with higher viscosity LM pectin.

The relative stability of gellan in both acellular (Figure 4.9b) and cellular conditions (Figure 4.9c) compared with LM pectin and to a greater extent alginate can be attributed to the very low concentrations of calcium required for gel formation. The concentration required to induce gelation of gellan with calcium cations is very low compared to that of alginate and LM pectin. Indeed, to give maximum gel strength, a

concentration roughly equivalent to the full stoichiometric requirement of the carboxyl groups on the polymer can be as low as ~3 mM for a gelling concentration of ~0.4 wt% gellan (Morris et al. 1996). In this study 100mM of CaCl_2 was used to crosslink 1% w/v gellan. Furthermore gellan hydrogels can be formed by exposure to monovalent cations unlike alginate and LM pectin therefore any cation exchange should not compromise gel strength as drastically as occurred in the other two hydrogels. Indeed, concentrations of cations in culture media alone have been previously shown to be sufficient to crosslink gellan (Smith et al. 2007). This is likely to be the cause of fluctuations in G' witnessed in the acellular gellan samples incubated in SDMEM (Figure 4.9b) with the changes corresponding to the introduction of fresh media when feeding the cells at different time points of the study. The cellular gellan gel samples degraded by ~20% of their original modulus by day 28 (Figure 4.8c). This degradation rate is similar to what was reported by Shoichet (1996) who reported that the gel strength of agarose diminished in the presence of cells on an average of 25% from day 0 to 60 before remaining constant until day 90 (Shoichet et al. 1996). This was explained by the interference of the cells with the hydrogen bond formation required for agarose gelation which could be an explanation for the increased rate of degradation in the cell loaded gels in the study. Moreover, the values for G' and G'' in the mechanical spectra at day 1 for the cell loaded samples (Figures 4.10a, 4.11a and 4.12a) were lower in all the gels compared with the acellular samples (Figures 4.10b, 4.10c, 4.11b, 4.11c, 4.12b and 4.12c). An interesting and more significant drop in G' was observed on day 14 in the cellular gellan samples which speculatively may correspond to a point in the culture prior to osteoblast differentiation where degradation is occurring due to utilisation of cations by the cells. The subsequent

recovery at day 21 in the gellan may therefore be due to a localised increase in calcium concentration supported by the formation of calcium phosphate mineral nodules (Figure 4.14b). This is more plausible in the gellan sample due to the very low concentrations required for gelation compared to concentrations required in alginate and LM pectin.

Interestingly, histological examinations of the rBMSC seeded hydrogels revealed osteoblastic mineralization in all three hydrogels (Figure 4.14a-c) therefore the changes in mechanical properties did not appear to impact on the cell phenotype however depending on the application of the construct the mechanical integrity could be critical. Even though all the cellular gels analysed at the completion of this study retained a gel like mechanical spectra ($G' > G''$ across a range of frequencies) (Figure 4.10b, 4.11b, and 4.12b) the differences in strength were apparent and provides an opportunity for designing hydrogel substrates which are application specific. The knowledge of the mechanical properties of the hydrogels is therefore important if a suitable scaffold for targeted tissue regeneration can be selected. For example the rapid degradation of alginate may be suitable for a cell delivery application but perhaps gellan would be more suitable for retaining cells in a desired location for longer periods.

4.5 CONCLUSION

This study has demonstrated that alginate, LM Pectin and gellan gum hydrogels can be used for the 3D culture of MSCs from which differentiation to osteoblasts can be achieved. The encapsulation of MSCs in these hydrogels resulted in degradation of the gels however this was at vastly different rates. An understanding of how mechanical

properties can vary over a relatively short cell culture period is important in selecting a suitable cell culture substrate. It has also highlighted the effect of cell culture media on the mechanical properties of the different ionotropic gels that also needs to be considered and the realization of this is very important.

5. RHEOLOGICAL MEASUREMENTS OF HA INCORPORATED IN GELLAN GUM HYDROGEL

5.1 INTRODUCTION AND AIM

Scaffold materials used in bone tissue engineering need mechanical strength to allow the device to be implanted without failing. Hydrogels can be reinforced with ceramics as in HA to fulfil this requirement. Near 60 wt% of human bone is made up of HA (LeGeros & LeGeros 2003). Investigations have characterized calcium phosphates such as HA which have close chemical and crystal resemblance to bone mineral to have excellent biocompatibility and osteoconductive activity (Gkioni et al. 2010;Yoshikawa & Myoui 2005). Recent studies by Tampieri (2005) and Parhi (2006) have reported a study on the improvement of the mechanical properties of alginate and HA composite crosslinked by Ca^{2+} ions (Parhi et al. 2006;Tampieri, et al. 2005). Another research by Pramanik investigated on the characterization and biocompatibility of HA/chitosan phosphate nanocomposite for tissue engineering applications by varying the HA content from 10 to 60% w/w (Pramanik et al. 2009b). Since then, there have been many more attempts to improve the mechanical strength of polymers with incorporation of ceramics as in HA (Pielichowska & Blazewicz 2010;Wei et al. 2011).

In the previous chapter, we demonstrated that a population of marrow stromal cells can be stimulated to deposit mineral within a hydrogel matrix. However, as the mechanical properties of the gel deteriorated in culture conditions it was impossible to determine whether the mineral deposition had a significant influence on the mechanical properties of the gel. In this chapter, we have loaded nanocrystalline HA similar to that found in the bone nodules into gellan hydrogel matrix in controlled

quantities. The objective of this work was to determine how mineral content influences the mechanical properties of a hydrogel based material. A recent study by Bouropoulos et al. (2010) has incorporated HA into alginate 2% w/v hydrogel crosslinked with 500mM CaCl_2 for the aim of reinforcement of the gel. The results obtained represented a range between 0.32-0.5wt% of HA to reinforce alginate gel (Bouropoulos et al. 2010). However this chapter involves an investigation on gellan gel in concentrations of 0.5%, 1% and 1.5% w/v crosslinked with 10mM CaCl_2 in the form of powder. Samples were then loaded with 50, 62.5 and 75mg/mL HA.

5.2 MATERIALS AND METHODS

Low-acyl Gellan Gum (Gelrite) was from Sigma-Aldrich Company Ltd., Dorset, UK, with an average MW of 1,000,000. Calcium chloride dihydrate was from Fisher Scientific. Unless specified, all other materials were obtained from Sigma-Aldrich Company Ltd., Dorset, UK.

5.2.1 PREPARATION OF POLYSACCHARIDE SOLUTIONS

The gellan gum polysaccharide solution was prepared by dissolving in deionized water (dH_2O) at 85°C with continuous stirring to a concentration of 0.5, 1 and 1.5% w/v. The prepared polysaccharide solution was autoclaved before used to make hydrogels.

5.2.2 GELLAN GUM GEL

Gellan gum was poured into disk-shaped moulds in 6-well plates for rheological tests and in bijoux tubes for compression tests, calcium chloride was added in powder form

(10 mM) to crosslink the gel while loading HA. Samples of 0.5% w/v, 1% w/v and 1.5% w/v of gellan gel were prepared with loading of three different concentrations of HA, 50 mg/mL, 62.5 mg/mL, and 75 mg/mL and cooled instantly with placing in a bucket of ice.

5.2.3 HA PREPARATION

HA was prepared by dissolving 132.06g (1 mole) of ammonium phosphate dibasic in 1.5L of dH₂O. The solution was diluted to 3.21L by the addition of dH₂O. Calcium nitrate tetrahydrate [Ca(NO₃)₂·4H₂O] 330.6g (1.4 moles) was dissolved in 900 mL of dH₂O. The mixture was diluted to 1.8L by addition of dH₂O. The pH of each of the resulting solutions was adjusted to 11 by the addition of concentrated aqueous NH₄OH at ambient temperature. The ammonium phosphate dibasic was added to calcium nitrate tetrahydrate using a Watson Marlow feeding pump (32 rpm) at a set speed of 9.95×10^{-9} m³/sec. The ammonium phosphate dibasic solution was stirred while it was being added to. The calcium nitrate solution (HA precipitate was being formed) was stirred at 375 rpm using a magnetic stirrer for 12 hours at 28.5°C. The synthetic HA was washed three times using EDTA (Ethylenediaminetetraacetic acid) and let dry for around 4 hours in the oven 100°C. The dried samples were then grounded using a pestle and a mortar. HA was sieved through a 212 µm mesh to ensure a uniform particle size distribution.

5.2.4 RHEOLOGICAL MEASUREMENTS

The mechanical properties exhibited by the hydrogels were determined using

oscillatory rheological measurements of storage modulus (G'), loss modulus (G''), and dynamic viscosity (η^*) at 37°C using a stress-controlled rheometer (TA instruments). The samples were characterized using 20 mm stainless steel parallel plate geometry (sand blasted to prevent slippage) at 0.5% strain, across a frequency (ω) range of 1 rad/s to 100 rad/s at a constant temperature of 37°C. Prior to loading, the gelled disk was trimmed to size using a razor blade and the thickness of the gels corresponded to the geometry gap (3mm). All tests were performed in triplicate, and mean values and corresponding standard deviations calculated.

5.2.5 COMPRESSION MEASUREMENTS

Cylindrical moulds were removed from the bijoux tubes and cut using a razor blade to achieve flat upper and lower surfaces and to bring the total specimen height to 30mm. The radius of the moulds was 15mm. Compressive testing of cylindrical specimens was carried out using Instron 5544 universal test machine operated at a crosshead speed of 5mm/min with 100N load cell. Samples were measured five times and the mean values and standard deviations of the stress-strain data at failure were calculated.

5.2.6 HA PARTICLE CHARACTERIZATION

Particle size distribution of aqueous dispersed HA was monitored in a ZETASIZER (NIBS TECHNOLOGY). Samples were measured three times and the mean value of the particle sizes was shown. The surface morphologies of the HA composite were observed using scanning electron microscopy (SEM) (XL30 ESEM FEG Electron

Microscope) and the transmission electron microscopy (TEM) (Technai F20 Philips). The HA was tested on a 400 Cu grid and the samples were dispersed in acetone for taking images.

5.2.7 CALCIUM RELEASE

The kinetics of HA dissolution were determined colourimetrically, measuring the absorbance intensity of the calcium sensitive dye arsenazo III (AIII). This dye changes colour upon binding to Ca^{2+} ($\text{Ca-AIII}\lambda_{\text{max}}=600 \text{ nm}$). Samples were prepared through dissolving 0.4 mM arsenazo in 100 mM of tris HCl buffer at pH 7.4. Calcium release was measured through the entrapment of HA within visking tubing (mwco 3,500) over a 24-hour period. Samples of absorbance were measured three times.

5.2.8 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used for statistical analysis, followed by a Tukey post-hoc test to determine the statistical significance ($p < 0.05$) between test groups (SPSS v.17, Chicago, USA).

5.3 RESULTS

5.3.1 ZETA AVERAGE PARTICLE SIZE

The average particle size distribution of HA was found to be 497.3 nm ($p < 0.05$).

HA Average Particle Size (nm) \pm SD: 497.3 \pm 19

The measurements have ensured even particle size distribution of HA within the samples. It has clarified the HA particle size distribution to be around 0.5 micron

which is in nano range. It is important to note that agglomeration in this size of filler is with a smaller possibility than nano crystal particles below the size of 100nm. However, higher concentration of HA could cause such phenomena in larger size particles (Shokrollahi P. 2008).

Particle size measurements enable greater control over keeping the system homogeneous and particles in suspension. In order to obtain high mechanical properties in gels, it is important to have homogenous distribution of HA particles.

5.3.2 TEM/SEM CHARACTERIZATION

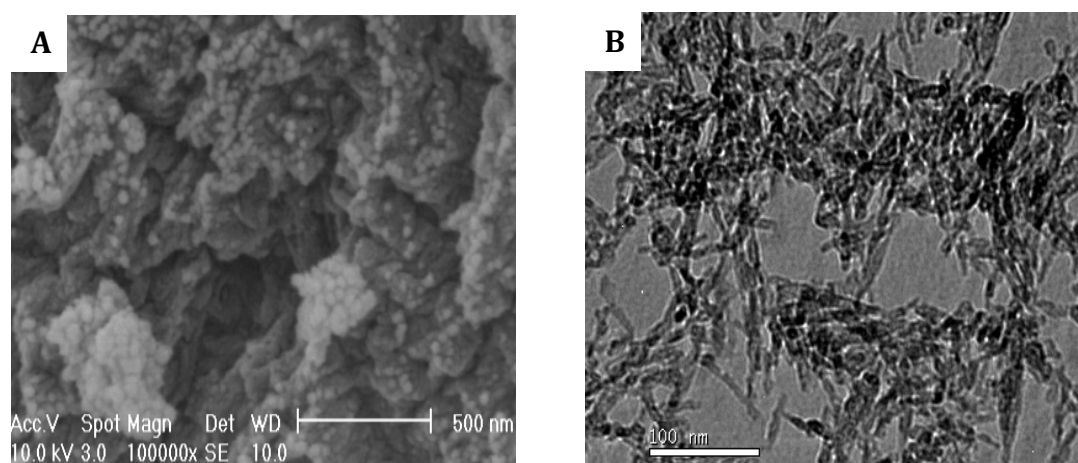


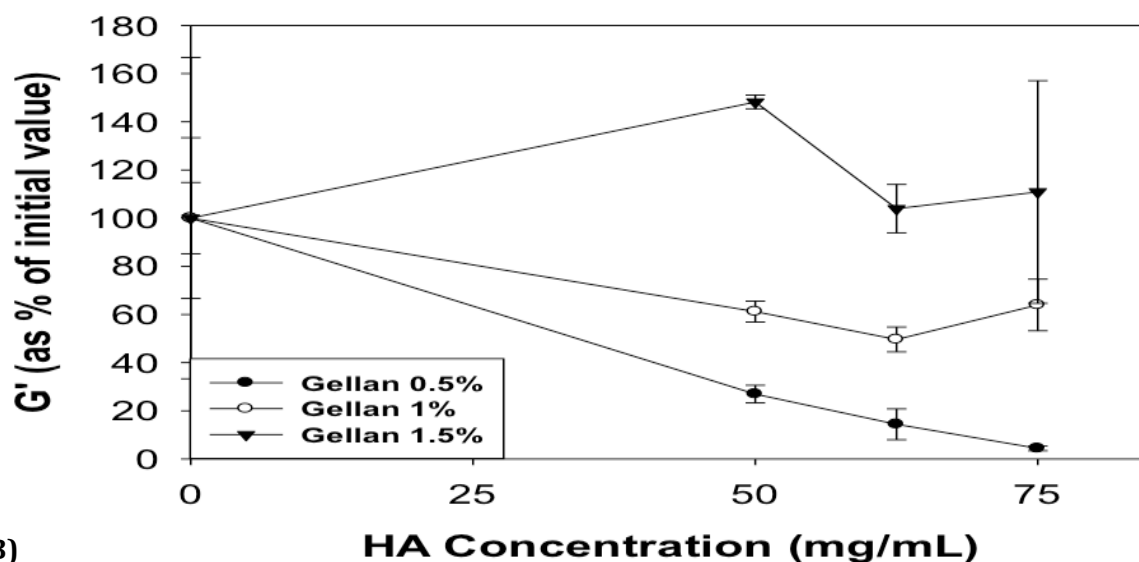
Fig. 5.1 SEM and TEM micrograph of HA particles (a) SEM micrograph, (b) TEM micrograph representing nano-crystalline HA

Distribution of filler particles in the polymer matrix was studied with scanning electron microscopy (SEM). Figure 5.1a depicts the particle size of nano-crystalline HA incorporated into gellan gel matrix. The clusters of HA are more or less in accordance with the results obtained for the particle diameter suggesting a uniform distribution of particles (Fig. 5.1a). The TEM images of the rod-like HA crystals (Fig. 5.1b) show the nano-crystalline HA particles to be long with rod-like crystals.

RHEOLOGICAL ANALYSIS

Rheological measurements of G' , G'' and η^* were taken to evaluate the mechanical behavior of HA loadings of 50mg/mL, 62.5mg/mL and 70mg/mL on 0.5% w/v, 1% w/v and 1.5% w/v gellan gel.

(A)



(B)

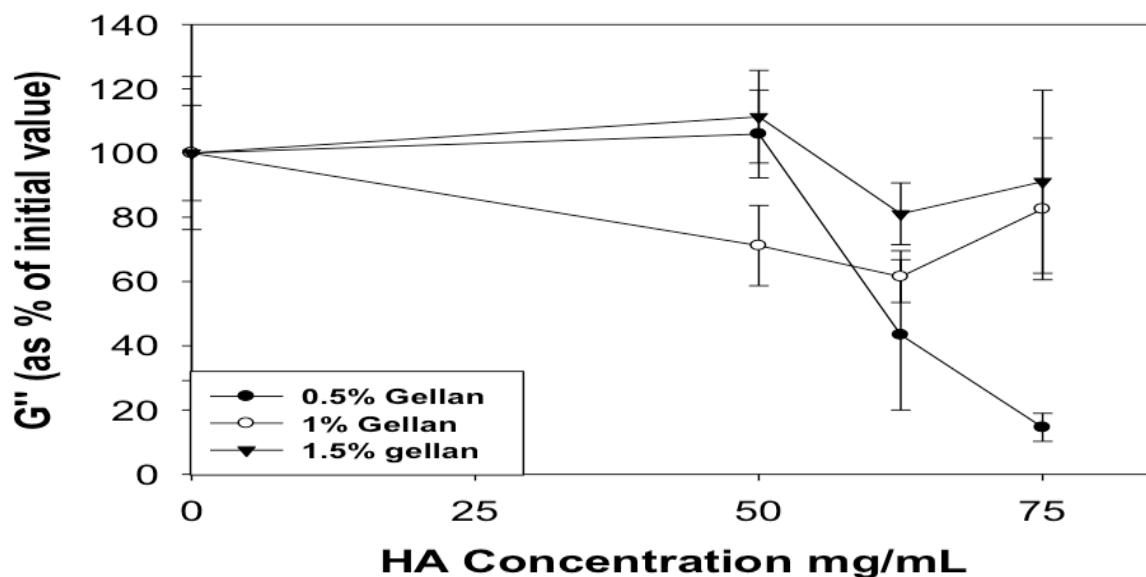


Fig. 5.2 Percentage change in (a) G' and (b) G'' (10 rad s^{-1} ; 0.5% strain; 37°C) of gellan samples with concentrations of 0.5% w/v (filled circles), 1% w/v (open circles) and 1.5%w/v (filled triangles) with HA loadings of 50mg/mL, 62.5mg/mL and 75mg/mL. Vertical error bars represent the standard deviation of the reported mean values ($n=3$).

The dynamic shear moduli G' and G'' of each concentration was measured and the changes in G' and G'' (measured at an angular frequency of 10 rad s^{-1} 0.5% strain 37°C) is represented in Figure 5.2 as a percentage of original modulus. Figure 5.2a reports a gradual decrease in G' of gellan at 0.5% as the amount of HA loading increased. Its modulus is dramatically dropped; to about 24% of its original value when 50mg/mL of HA was added. A further reduction in the gel strength is experienced as 62.5mg/mL of HA is added and this trend continues with the addition of 75mg/mL. However, in the gel concentration of 1% w/v, less loss of modulus is experienced. Incorporation of 50mg/mL of HA resulted in 32% drop in the gel modulus. Addition of 62.5mg/mL of HA resulted in further 45% decrease in the modulus of the gellan gel. Incorporation of 75mg/mL HA in gellan resulted in a stronger gel with increased modulus to around 90% of its original value.

1.5% w/v gellan gel behaves slightly different. Addition of 50mg/mL HA increased the gel strength by about 60% of the original value and further addition of HA (62.5mg/mL) decreased the achieved modulus back to its original value. Further loading of 75mg/mL HA did not show an increase in the mechanical properties of the gel ($p < 0.05$). As Figure 5.2b represents G'' , it is evident that in 0.5% w/v concentration, the gellan modulus drops with the addition of HA. Concentrations of 1% and 1.5% w/v followed an almost similar path, decreasing with incorporation of 62.5mg/mL HA and only slightly increasing with the addition of 70mg/mL HA.

5.4 COMPRESSION ANALYSIS

Gellan gel strength and ultimate failure in the presence of HA has further been characterized through compression testing. Figure 5.3 measures stress and strain at

failure point of gellan gel samples through compression tests performed at different loadings of HA.

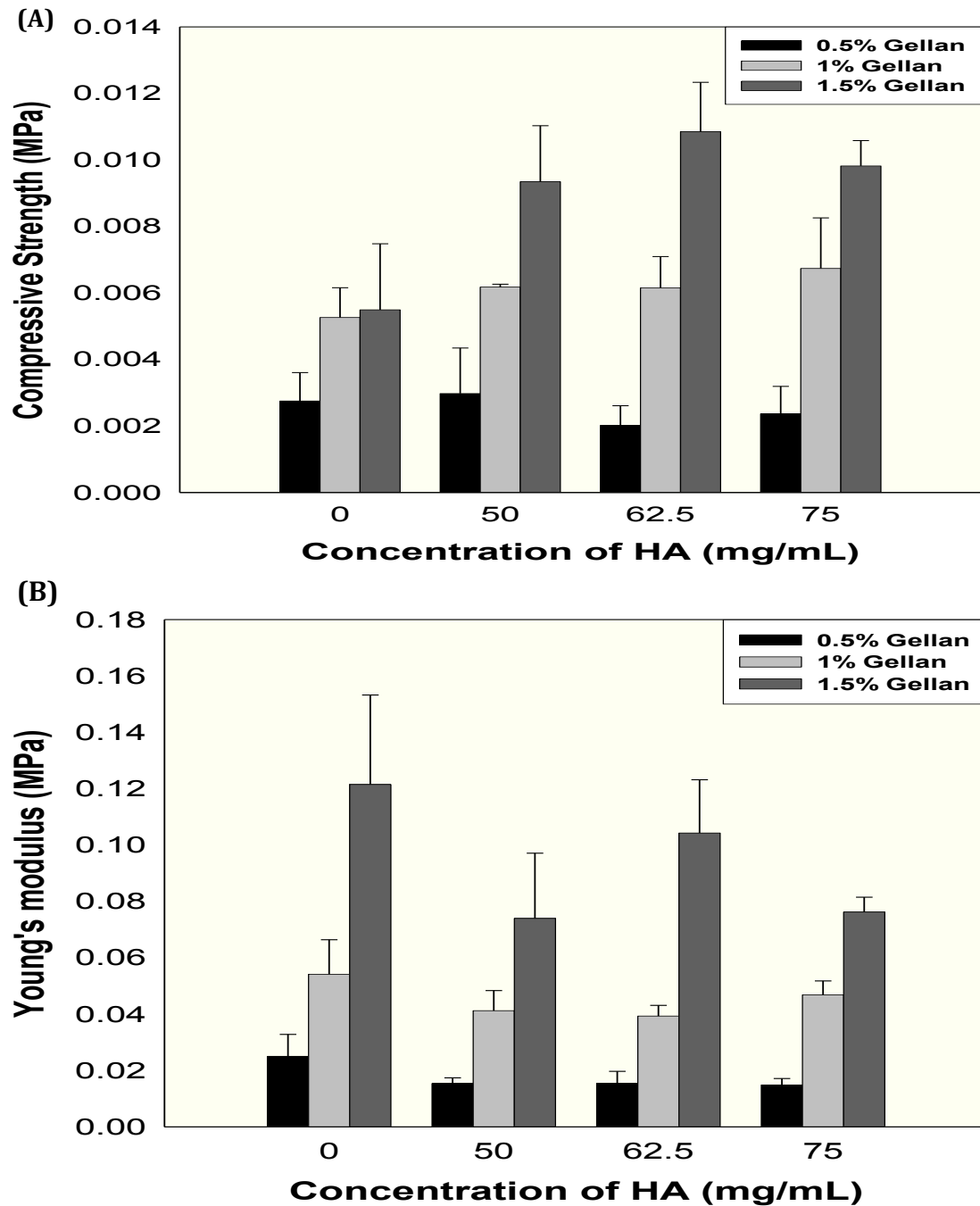


Fig. 5.3 (a) Compressive strength at failure (b) Young's modulus of 0.5% w/v, 1% w/v and 1.5% w/v gellan gels with loadings of 0mg/mL, 50mg/mL, 62.5mg/mL and 75mg/mL of HA. Vertical error bars represent the standard deviation of the reported mean values (n=5).

Addition of 50mg/mL, 62.5mg/mL and 75mg/mL of HA in three concentrations of 0.5% w/v, 1% w/v and 1.5% w/v gellan gel has made a comparison between the three concentrations. The compressive strength of 0.5% w/v gellan samples in Figure 5.3a has shown to be 2.7kPa which has thoroughly decreased by around 2kPa with an increase in the amount of HA in the gel. The data further represents a compressive strength of 5.2kPa in 1% w/v gellan gel without HA with a slight increase of about 1.5kPa throughout the modulus graph with maximum amount of 75mg/mL HA. 1.5% gellan gel increases strength by around 3.9kPa with incorporation of 50mg/mL HA into the gel, and presents an optimum compressive strength of 10.9kPa at 62.5mg/mL HA loading. Following this, the compressive strength falls by about 1KPa with 75mg/mL HA loading.

The Young's modulus presented in Figure 5.3b is an indication of the stiffness of gellan gel. A thoroughly decrease in the stiffness of 0.5% w/v gellan samples is observed as the HA loading has increased. At this concentration, the Young's modulus is presented at 25kPa with no HA in the polymer and decreases to around 15kPa with 75mg/mL HA. The gel samples of 1% w/v show a stiffness of 54kPa at no HA/gel falling to around 39kPa with incorporation of 62.5mg/mL HA. The stiffness has slightly increased by 7kPa with increasing the amount of HA to 75mg/mL. The samples with highest original stiffness of 1.5% w/v gel show the highest Young's modulus value of 121kPa. The Young's modulus fluctuates as the amount of HA is increased. Addition of 50mg/mL of HA to the samples, decreased the stiffness by about 47kPa. Further increase in the amount of HA enhanced the modulus to 104kPa and again lost stiffness of about 28kPa in samples with 70mg/mL HA.

5.4.1 CALCIUM RELEASE

Further to our investigations, the amount of Ca^{2+} released from HA as a calcium source was measured over 24 hours period. Measurements were taken every 10 minutes within the first hour followed by readings at 2, 3 and 24 hours. Figure 5.4a reports that around 45% of Ca^{2+} were released in the first 3 hours of the study and the rest was gradually released throughout the 24 hours study (Figure 5.4b).

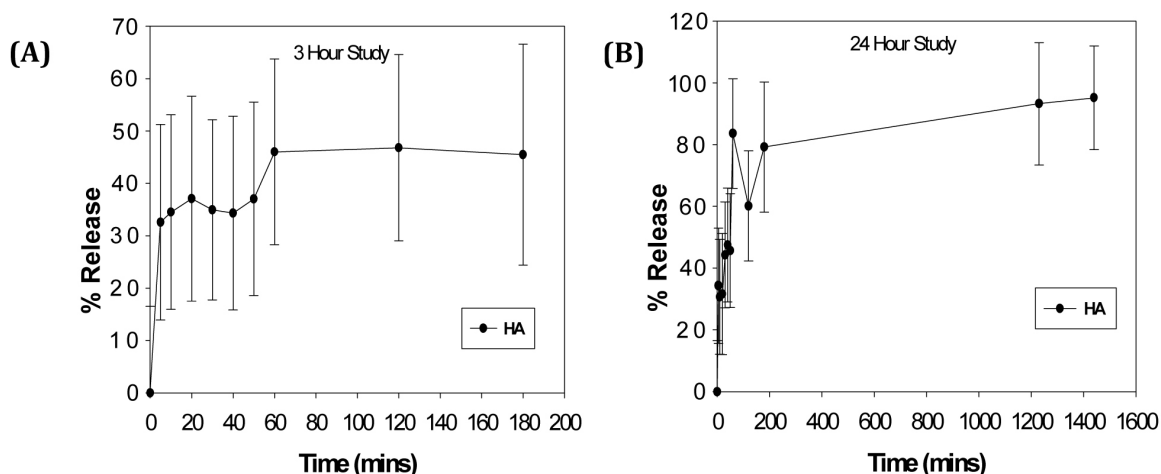


Fig. 5.4 Percentage release of Ca^{2+} from HA over a period of 3 and 24 hours. (a) Percentage release over 3 hours (b) percentage release over 24 hours. Vertical error bars represent the standard deviation of the reported mean values (n=3).

5.5 DISCUSSION

In general, incorporation of osteoconductive bioceramics enhances the elastic modulus and biocompatibility of the subsequent composites (Kim et al. 2004). Yet, there is a critical inorganic loading limit, which above that, the mechanical strength starts to decrease (Yang et al. 2009). Recent studies have identified nano-HA (0-100 nm) as a reinforcement agent in biocomposites like chitosan, agarose and alginate (Pramanik et

al. 2009a;Watanabe et al. 2007;Lin & Yeh 2004). This study further reports the mechanical reinforcement of HA/gellan biocomposite. The nanocrystalline HA used in this study has an average particle size distribution of around 0.5 micron, which is well above ideal 1-100nm nano-particle size. Therefore, the effect of agglomeration (high specific surface energy of nano-HA particles resulting in their aggregations) could not possibly have had an effect on the results observed from the modulus of the gel. The rheological measurements (Figure 5.2) report a decrease in the modulus with addition of HA to the gel matrix at 0.5% w/v gellan concentration. 1% w/v gellan concentration relatively shows a decrease in the modulus but to a lesser extent. This is while, 1.5% w/v concentration stiffens with incorporation of 50mg/mL HA and then returns to original modulus. The overall difference in the modulus of varying concentration of the gel could be related to the available chemical bonding sites in higher concentration of gels compared to the lower concentrations that have resulted to an increase in the modulus of the higher concentrations. In general, the tetrasaccharide network of gellan has both free carboxylic and hydroxylic groups. The free OH^- ions of gellan form hydrogen bonds with the free unstable Ca^{2+} ions released from HA and CaCl_2 crosslinker. Also, the phosphate group of HA favor hydrogen bonds with carboxylic group of gellan gel forming and acid-base interaction. The overall decrease in the modulus observed in Figure 5.2 could be related to the large particle size distribution of 497nm in high amount compared to gellan gel concentration causing agglomeration of HA clusters which could result in decrease of the modulus of the gel hampering the intended improvement in strength of the gel.

The results of the mechanical measurements in uniaxial compression mode (Figure 5.3a) showed almost the same trend as the dynamic rheological measurements in

0.5% w/v gellan gel. However, the strengthening effect of the bioceramic on the mechanical properties of the composite at 1% w/v and 1.5% w/v can be seen in this figure with an optimum concentration of 62.5mg/mL HA in 1.5% w/v gellan. The Young's modulus (Figure 5.3b) also decreased at 0.5% w/v gellan. In 1% w/v gellan gel, the modulus fluctuated and the gel slightly lost its stiffness. Optimum reinforcement was obtained in 1.5% w/v gellan gel with incorporation of 62.5mg/mL HA. The mechanical properties gradually decrease above this limit that can be attributed to the amount of HA compared to the gel forming clusters of HA in the composite disrupting the network structure.

The amount of Ca^{2+} released by HA within the first 3 hours (Figure 5.4) in the calcium release study suggests that HA on its own releases enough calcium to physically crosslink gellan without any chemical compounds such as CaCl_2 . This of course is beneficial to the cells.

5.6 CONCLUSION

In this study, we prepared a novel HA/gellan composite and investigated the effect of this bioactive ceramic on the mechanical properties of gellan at three concentrations. We observed that HA particle size of around 0.5 micron does not have a strong effect in reinforcement of lower concentration gellan gel. This effect is more apparent in smaller size particles (1-100 nm) with higher surface area to volume ratio. The larger size particles could form clusters of HA and agglomeration leads to failure initiation at the interface between the matrix and the agglomerated particle. Therefore, the

modulus of the gel reduces. Calcium release study proved that HA releases enough Ca^{2+} to physically crosslink and so chemical crosslinkers are no longer needed in the presence of HA.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 SUMMARY AND OVERALL CONCLUSIONS

In this thesis, the change in mechanical properties of three biopolymer hydrogels alginate, LM pectin and gellan gum seeded with BMSCs have been assessed in cell culture conditions *in vitro*. The results obtained proved that the hydrogels are suitable to be used for the 3D culture of BMSCs from which differentiation to osteoblasts can be achieved. However, the rate of degradation of the hydrogels varied vastly, alginate appearing with the highest rate of degradation in culture media and gellan gel the least. This makes each suitable for culture of specific target tissues. The mineral deposition obtained through osteoblastic differentiation was hypothesized to increase the mechanical strength of the gel. However due to the rapid degradation of the hydrogels, it was impossible to further investigate this on the same samples.

Further to this study, the influence of mineral depositions on the mechanical properties of gellan gum hydrogel was investigated with incorporation of nanocrystalline HA into the gel. It was evidential that incorporation of HA increased the hydrogel stiffness in higher concentrations. This was supported by the rheological and compression tests performed. In addition, the results obtained from the calcium release experiment on the amount of calcium released from HA over 24 hours indicated that enough calcium is available for physical crosslink of gellan. Therefore, in the presence of natural minerals as in HA, there is no need for chemical crosslinking and this is beneficial for the cells.

These results highlight the mechanical properties of biopolymer hydrogels that vary greatly during *in vitro* culture without having an impact on the rBMSCs differentiation. It therefore provides the potential of selecting hydrogels as cell culture substrates with mechanical properties that are tissue specific. Also the importance of particle size for reinforcement of HA/gellan composite is highlighted at varying concentrations.

6.2 FUTURE PERSPECTIVES

One of the major challenges in bone tissue engineering is the production of a suitable scaffold material. Biomaterial and bone tissue engineering studies beyond those cited offer important perspectives on the opportunities and challenges that lie ahead. Although this thesis demonstrated that alginate, LM pectin and gellan gum hydrogel are suitable scaffolds to be used in 3D culture of BMSCs from which differentiation to osteoblasts was achieved and investigated on the effect of mineralization on the modulus of gellan gel via incorporation of HA, It is important to further assess these potentials.

6.2.1 DEGRADATION OF POLYSACCHARIDE HYDROGELS IN CELL CULTURE CONDITIONS

Further work should seek to quantify the amount of type I collagen and alkaline phosphatase in differentiated osteoblasts during matrix deposition. In addition, in order to improve the mechanical strength, degradation rate and osteoconductivity, gels can be combined with nano-particles of bioceramics such as β -tricalcium phosphate, different ratios of HA, demineralized bone matrix or calcium carbonate

(Weinand et al. 2006;Tang et al. 2009). Moreover, hydrogels can be modified to exhibit biochemical, cellular and physical stimuli that guide cellular processes of migration, proliferation and differentiation. This can be achieved by the addition of biologically active additives such as bone morphogenic protein (BMP), growth factors, osteoblasts and more (Lutolf et al. 2003;Kodama et al. 2009;Wang et al. 2009).

6.2.2 RHEOLOGICAL MEASUREMENTS OF HA INCORPORATED IN GELLAN GUM HYDROGEL

Addition of biodegradable and bioactive ceramics as in HA to the polymer scaffolds used for applications in bone reconstruction has many advantages. This work should systematically investigate the optimum amount of HA for different concentrations of gellan gel and should seed the biocomposite with BMSCs to observe the effect of bone mineralization coupled with HA content on the matrix modulus. It is also important to control factors as in adhesion of HA to the gellan gel structure by covalent attachment of collagen-I to increase osteoblast differentiation and reinforce mineralization.

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8. APPENDIX

- JOURNAL PUBLICATION

Jahromi, S.H., et al., Degradation of polysaccharide hydrogels seeded with bone marrow stromal cells. Journal of the Mechanical Behavior of Biomedical Materials (2011), doi:10.1016/j.jmbbm.2011.03.025

- OTHER RESEARCH ATTRIBUTIONS

Smith A.M., Kermali I., **Jahromi S.H.**, Grover L.M. (2010) Particulate reinforcement of alginate hydrogels using nanocrystalline hydroxyapatite. UK Society for Biomaterials Annual Conference. Glasgow, UK, Oral presentation.

Jahromi S.H., Smith A.M., Grover L.M. (2010) The degradation behaviour of biopolymer gels under cell culture conditions. UK Society for Biomaterials Annual Conference. Glasgow, UK, Poster

Smith A.M., **Jahromi S.H.**, Grover L.M. (2010) A systematic study of the degradation behaviour of biopolymer gels under cell culture conditions. Proceedings of the 10th International Hydrocolloids conference. Shanghai, China, Oral Presentation